The role of NF-κB signalling in T cell homeostasis and function

Louise Victoria Webb

UCL

A thesis presented for the degree of Doctor of Philosophy, 2015

Statement of Declaration

I, Louise Victoria Webb, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Louise Victoria Webb

Acknowledgements

Firstly, I wish to thank my wonderful supervisor, Dr. Benedict Seddon. Ben is one of those very special people whose academic brilliance is matched by his positive attitude, calmness, and kindness. Ben, you have made my PhD experience very enjoyable and, for that, I shall always be grateful.

> *"To be creative, scientists need libraries and laboratories and the company of other scientists; certainly a quiet and untroubled life is a help. A scientist's work is in no way deepened or made more cogent by privation, anxiety, distress, or emotional harassment. To be sure, the private lives of scientists may be strangely and even comically mixed up, but not in ways that have any special bearing on the nature and quality of their work."*
>
> Peter Medawar
> In "Advice to a Young Scientist" (Medawar, 1979)

Next, I would like to thank all those previous lab members for their help and encouragement. Ana, thank you for teaching me so many new techniques during my first few months, you were so kind and patient. Iren, thanks for developing a computer code to make my calculation of cell numbers so much easier. Charles, thanks for introducing me to RNA sequencing, it proved very useful. Sim, thanks for being such a lovely technician. Dan and Ina, my fellow students, thanks for all your guidance.

Now, to the current members of the Seddon lab, I extend my heartfelt thanks and appreciation. Thea, you have been present throughout my PhD. Your FACS knowledge is amazing, and you always have wonderful suggestions. Bea, thanks so much for all your hard work setting up the new lab at the Royal Free Hospital. You are so organized and always know just what needs to be done. Alessandro, I am so glad that we can talk about NF-κB signalling together. Thanks for teaching me your western blotting technique. Jie, I cannot thank you enough. I really value the time that we have spent together at the NIMR. You have done so much for me - you are truly one in a million.

Thanks also to my wonderful, supportive family. Especially to Mum, Dad, and my sister, Katy. You always encouraged me to follow my interests. I could not have asked for a better family. I love you all so much.

Finally, I would like to thank everyone at the MRC's National Institute for Medical Research (NIMR). I feel so proud to have been a part of this historical institute during its very final years. Perhaps not everyone would agree, but, to me, there are few places as romantic as the NIMR with its tarnished green copper roof and corridors in the shape of an IgA molecule. I feel so privileged to have walked those same corridors once occupied by great immunologists such as Peter Medawar and his former student and my hero, Leslie Brent. For within the NIMR, I could not help but feel that I was walking in the footsteps of giants.

Abstract

Inhibitor of NF-κB kinase 1 (IKK1) and 2 (IKK2) form part of the functional IKK complex, necessary for NF-κB signalling. NF-κB is vital for survival, development, and function of multiple cell types. The aim of this study was to determine the function of NF-κB in thymic development and maintenance of the peripheral T cell compartment. For this, we used the Cre-loxP system in order to delete one or both of the *Ikk1* and *Ikk2* genes in T cells.

Developmental deletion of *Ikk1* and *Ikk2* in CD4 CD8 double negative thymocytes revealed that NF-κB signalling was, surprisingly, redundant for early stages of thymic development and selection. Instead, late stage maturation of single positive thymocytes was abruptly halted in the absence of *Ikk1* and *Ikk2*. Double deficient mice had a severe reduction in mature HSA low single positive thymocyte numbers and scarcely any peripheral T cells. Members of the TNF receptor superfamily (TNFRSF) are potent activators of NF-κB. We therefore tested the hypothesis that TNFRSF signalling could be regulating developing thymocytes. *In vivo* blockade of TNF in the IKK1/2 double deficient animals resulted in an almost complete rescue of thymocyte development. Cell culture experiments revealed that IKK1/2 double deficient thymocytes were specifically susceptible to TNF induced death. TNF receptor 1 (TNFR1) was identified as the key surface receptor, and TNFR1 IKK1/2 triple deficient mice had normal thymic development. Although TNFR1 is expressed throughout thymic development, transcriptomic analysis revealed cIAP2 and IL-7Rα as key targets of NF-κB dependent TNF signalling, but that co-operative developmental regulation of RIPK1 and caspase-8 underpinned the developmental susceptibility of mature SP thymocytes to TNF induced apoptosis.

Our data reveal that developmental tuning of TNFR signalling and response genes is critical for normal T cell development and homeostasis.

Table of contents

List of figures

List of schematics

List of tables

List of abbreviations

Abbreviations used in this thesis:

-	Negative/missing allele
-ve	Negative
+	Positive/allele present
±	With or without
*****	Significant at P<0.05
****	Significant at P<0.01
*****	Significant at P<0.001
4-1BBL	4-1BB ligand
A1/Bfl1	Bcl-2-related protein A1
Ab	Antibody
AB-IMDM	Air-buffered Iscove's Modified Dulbecco's Medium
ACK	Ammonium-Chloride-Potassium
ADCC	Antibody dependent cellular cytotoxicity
AIRE	Autoimmune regulator
AP-1	Activation protein-1
Apaf-1	Apoptotic protease activating factor-1
APC	Allophycocyanin
APC	Antigen-presenting cell
APC-eF780	Allophycocyanin e-Fluor 780
APECED	Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy
APRIL/TNFSF13	A proliferation-inducing ligand
BAFF-R	B cell activating factor receptor
BAFF/TNFSF13B	B cell activating factor of the tumour necrosis family
Bcl-2	B cell leukaemia/lymphoma 2
Bcl-10	B cell leukaemia/lymphoma 10
Bcl6	B cell leukaemia/lymphoma 6

BCR	B cell receptor
BD	Beckton Dickinson
BH	Bcl-2 homology
Bio	Biotin
BIR	Baculovirus IAP repeat
BSA	Bovine serum albumin
c-FLIP	Cellular FADD-like IL-1β-converting enzyme-inhibitory protein
C57BL/6	C57 black 6
Ca^{2+}	Calcium ions
CAD	Caspase-activated DNAse
CARD	Caspase activation and recruitment
CARMA1/CARD11	CARD-containing MAGUK protein 1
Caspase	Aspartic-acid-specific cysteine protease
Cbl-b	Casitas B-lineage lymphoma-B
CBM	CARMA1/Bcl10/MALT1
CCL	Chemokine C-C motif ligand
CCR	Chemokine receptor
CD	Cluster of differentiation
CD27L/CD70	CD27 ligand
CD30L	CD30 ligand
CD40L	CD40 ligand
cDNA	Complementary deoxyribonucleic acid
cIAP	Cellular inhibitor of apoptosis protein
CO_2	Carbon dioxide
Conc.	Concentration
Cre	Cre recombinase
cSMAC	Central supramolecular activation complex
cTEC	Cortical thymic epithelial cell
CTL	Cytotoxic lymphocyte
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
CXCL	Chemokine C-X-C motif ligand
CYLD	Cylindromatosis
D	Diversity gene segment

DAG	Diacylglycerol
DC	Dendritic cell
DD	Death domain
DED	Death effector domain
dH_2O	Distilled water
DISC	Death signalling inducing signalling complex
DMSO	Dimethyl sulfoxide
DN	Double negative
DNA	Deoxyribonucleic acid
DP	Double positive
DPBS	Dulbecco's phosphate buffered saline
DR	Death receptor
DTH	Delayed type hypersensitivity
EDA-A	Ectodysplasin A
EDTA	Ethylenediaminetetraaceticacid
eF450	e-Fluor 450
ER	Endoplasmic reticulum
Erk	Extracellular signal-regulated kinase
ETP	Early T cell progenitor
EYFP	Enhanced yellow fluorescent protein
F5	F5 *Rag1*$^{-/-}$
FACS	Fluorescence activated cell sorting/flow cytometry
FADD	Fas-associated protein with death domain
FasL	Fas ligand
FCS	Fetal calf serum
FDC	Follicular dendritic cell
FITC	Fluorescein isothiocyanate
fMet-Leu-Phe	N-formyl-methionylleucyl-phenylalanine
FoxP3	Forkhead box P3
fx	Floxed
G_1	Growth 1/Gap 1
GDP	Guanosine diphosphate
GITR	Glucocorticoid-induced TNF receptor

GITRL/TNFSF18	Glucocorticoid-induced TNF receptor ligand
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GRAIL	Gene related to anergy in lymphocytes
GTP	Guanosine triphosphate
H_2O	Water
HEV	High endothelial venule
hi	High
HOIL-1	Heme-oxidised iron regulatory protein 2 ubiquitin ligase 1
HOIP	HOIL-1 interacting protein
HSA/CD24	Heat stable antigen
Hsp90	Heat shock protein 90
huCD2	Human CD2
HVEM	Herpes virus entry mediator
I.P.	Intraperitoneal
IC_{50}	Concentration required for 50% inhibition
ICAM	Intercellular adhesion molecule
ICOS	Inducible co-stimulator
ICOSL/B7-H2	Inducible co-stimulator ligand
iCre	Codon-improved Cre recombinase
IFN	Interferon
Ig	Immunoglobulin
IKK deficient	Deficient in both IKK1 and IKK2
IKK	Inhibitor of NF-κB (IκB) kinase
IKK-K	IKK kinase
IKK1/IKKα	Inhibitor of NF-κB (IκB) kinase 1
IKK2/IKKβ	Inhibitor of NF-κB (IκB) kinase 2
$IKK\Delta T^{CD2}$	$huCD2^{iCre}$ *Ikk1*$^{fx/fx}$ *Ikk2*$^{fx/fx}$ $R26R^{EYFP}$
$IKK\Delta T^{CD4}$	$CD4^{Cre}$ *Ikk1*$^{fx/fx}$ *Ikk2*$^{fx/fx}$ $R26R^{EYFP}$
IL	Interleukin
IL-12R	Interleukin-12 receptor
IL-1R	Interleukin-1 receptor
IL-23R	Interleukin-23 receptor

IL-2R	Interleukin-2 receptor
IL-7R	Interleukin-7 receptor
IL-7Rα/CD127	Interleukin-7 receptor alpha chain
int	Intermediate
IP_3	1,4,5-trisphophate
IRAK	Interleukin-1 receptor-associated kinase
ISP	Immature single-positive
ITAM	Immunoreceptor tyrosine-based activation motif
Itk	Interleukin-2 tyrosine kinase
IκB	Inhibitor of NF-κB
J	Joining gene segment
JAK	Janus kinase
JNK	JUN N-terminal kinase
K48	Lysine 48
K63	Lysine 63
$KHCO_3$	Potassium hydrogen carbonate
KLH	Keyhole limpet hemocyanin
LAT	Linker of activated T cells
Lck	Lymphocyte-specific protein tyrosine kinase
LCMV	Lymphocytic choriomeningitis virus
LFA-1	Lymphocyte function-associated antigen-1
LIGHT/HVEM/TNFSF14	Ligand for herpes virus entry mediator and lymphotoxin receptor
LIGHTR	LIGHT receptor
LN	Lymph node
lo	Low
log	Logarithm
LPS	Lipopolysaccharide
LT	Lymphotoxin
LTβR	Lymphotoxin-beta receptor
LUBAC	Linear ubiquitin chain assembly complex
M-CSF	Macrophage colony-stimulating factor
mAb	Monoclonal antibody

MAL/TIRAP	MyD88 adapter-like
MALT1	Mucosa-associated lymphoid tissue lymphoma translocation protein 1
MAP2K	Mitogen-activated protein kinase kinase
MAP3K	Mitogen-activated protein kinase kinase kinase
MAPK	Mitogen-activated protein kinase
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MIP-3β/CCL19	Macrophage inflammatory protein-3 beta
MLKL	Mixed lineage kinase like
MRC	Medical Research Council
mRNA	Messenger ribonucleic acid
mTEC	Medullary thymic epithelial cell
MyD88	Myeloid differentiation primary response gene 88
NEMO/ IKKγ/ IKKAP	NF-κB essential modulator
NF-κB	Nuclear factor kappa B/nuclear factor of kappa light polypeptide gene enhancer in B cells
NFAT	Nuclear factor of activated T cells
NGS	Next-generation sequencing
NH_4Cl	Ammonium chloride
NIK	NF-κB-inducing kinase
NIMR	National Institute for Medical Research
NK	Natural killer cell
NLR	NOD-like receptor
no.	Number
NODD-LRR	Nucleotide-binding oligomerization domain leucine rich repeat
NP	Influenza virus nucleoprotein
NP40	Nonidet P-40
ns	Not significant
OVA	Ovalbumin

OX40L	OX40 ligand
PAMP	Pathogen associated molecular pattern
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE Cy5	Phycoerythrin cyanine 5
PE Cy7	Phycoerythrin cyanine 7
PE	Phycoerythrin
PE TR	Phycoerythrin Texas red
PerCP Cy5.5	Peridinin-chlorophyll protein cyanine 5.5
PH	Pleckstrin homology
PIP_2	Phosphatidylinositol bisphosphate
PIP_3	Phosphatidylinositol 3, 4, 5-trisphosphate
PKC-θ	Protein kinase C-theta
PLC- γ	Phospholipase C-γ
pLck	Proximal Lck
PO	Pacific orange
PRR	Pattern recognition receptor
PSG	L-glutamine penicillin-streptomycin
pSMAC	Peripheral supramolecular activation complex
pTα	Pre-T alpha chain
R26R	Rosa 26 reporter
rad	Unit of absorbed radiation dose
RAG	Recombination activating gene
RANKL	RANK ligand
Red-IETD-FMK	RED-IETD-fluoromethyl ketone
RHD	Rel homology domain
RING	Really interesting new gene
RIP	Receptor interacting protein
RIPK1/RIP1	Receptor interacting protein kinase 1
RIPK2/RIP2	Receptor interacting protein kinase 2
RIPK3/RIP3	Receptor interacting protein kinase 3
RLR	RIG-I like receptor
RNA	Ribonucleic acid

RORγt	RAR-related orphan receptor gamma thymus
RPMI 1640	Roswell Park Memorial Institute 1640
RTE	Recent thymic emigrant
RunX3	Runt-related transcription factor 3
S1P	Sphingosine-1-phosphate
$S1P_1$	Sphingosine-1-phosphate receptor 1
SAV	Streptavidin
SEM	Standard error of the mean
SH2	Src homology 2
SHARPIN	SHANK-associated RH domain interactor
SLC/CCL21	Secondary lymphoid chemokine
SLE	Systemic lupus erythematosus
SLP-76	SH2 domain-containing leukocyte protein of 76kDa
SMAC	Supramolecular activation complex
Smac/DIABLO	Second mitochondria-derived activator of caspases
SOCS	Suppressor of cytokine signalling
SODD	Silencer of death domain
SP	Single positive
SPF	Specific pathogen free
STAT	Signal transducer and activator of transcription
TACE	TNF alpha converting enzyme
TACI	Transmembrane activator and CAML interactor
TAK1	TGF-β activated kinase 1
TCR	T cell receptor
Tfh	T follicular helper cell
TGF-β	Transforming growth factor beta
Th	T helper cell
ThPOK	T helper inducing POZ-Kruppel factor
TIR	Toll-IL1R
TLA1/VEGI/TNFSF15	TNF-like protein 1A

TLR	Toll-like receptor
TNF	Tumour necrosis factor
TNFR	Tumour necrosis factor receptor
TNFR1/CD120a	Tumour necrosis factor receptor 1
TNFR2/CD120b	Tumour necrosis factor receptor 2
TNFRSF	Tumour necrosis factor receptor superfamily
TNFSF	Tumour necrosis factor superfamily
tpA	Strong transcriptional termination sequence
Tr1	Type 1 regulatory
TRADD	TNFR1 associated death domain protein
TRAF	TNF receptor associated factor
TRAIL/TNFSF10	TNF-related apoptosis-inducing ligand
TRAM	TRIF-related adapter molecule
T_{reg}	Regulatory T cell
TRIF	TIR-domain-containing adapter-inducing interferon beta
TWEAK/TNFSF12	TNF-related weak inducer of apoptosis
V	Variable gene segment
VCAM-1	Vascular cell adhesion protein-1
VLA-4	Very late antigen-4
WT/wt	Wild-type
XIAP	X-chromosome linked inhibitor of apoptosis
XL-EDA-ID	X-linked anhidrotic ectodermal dysplasia with immunodeficiency
YFP	Yellow fluorescent protein
Z-FA-FMK	Benzyloxycarbonyl-phenyl-alanyl-fluoromethyl ketone
Z-IETD-FMK	Z-IETD-fluoromethyl ketone
Zap70	Zeta-chain-associated protein kinase 70
αCD27L	Anti-CD27 ligand
αTNF	Anti-TNF
γ_c	Common γ-chain

Units used in this thesis:

%	Percent
g	Relative centrifugal force
hr	Hour
kDa	Kilodalton
mg	Milligram
min	Minute
mL	Millilitre
mM	Millimolar
ng	Nanogram
nRPKM	Number of reads per kilobase of exon per million sequenced reads
^{o}C	Degrees Celsius
v/v	Volume/volume
w/v	Weight/volume
µg	Microgram
µL	Microlitre
µm	Micrometre
µM	Micromolar

Chapter 1 Introduction

1.1 A brief history of Immunology

Immunology is a relatively new discipline. Throughout its short lifespan, periods of rapid advance have been interjected with those of slow progress. Over the years, immunology has been hugely influenced by international politics, with many of its main discoveries taking place in the aftermath of the Franco Prussian War of 1870 and of both World Wars. Topics have been hugely and sometimes fiercely debated. Often too much attention seems to have been focused on one area with the subsequent neglect of the other. Today our understanding of the immune system is often accredited to a handful of very successful individuals – the heroes or “giants” of immunology. Yet, remarkable progress has been made in a short space of time and, for that, we have more than a few dedicated immunologists to thank.

1.1.1 Early understanding of disease and immunity

Hippocrates, the Greek physician born around 460 BC, was the first to suggest that disease may not be entirely theurgic in nature. He described the existence of the four humors – blood, phlegm, yellow bile, and black bile. A humoral imbalance, it was believed, could account for all manner of maladies. Practices involving the use of leeches, cupping, purgatives, and phlebotomy became standard procedure (Silverstein, 2012).

Somewhat remarkably, very little progress was made over the next two millennia and the origin of disease remained elusive. Nevertheless, the idea of disease as a contagious entity and the concept of immunity as something to be acquired did slowly become established. In 1714 Emanuele Timoni and Jacob Pylarini presented, to the Royal Society of London, the concept of variolation. This was the practice of taking material from a patient infected with smallpox and introducing it into a healthy individual. The aim was to produce a less-severe form of the disease, which, once overcome, would confer on the patient

a lasting immunity (Silverstein, 2012). This practice was not new, having been used frequently in Eastern medicine. It was, however, met with distrust among the peoples of London and Paris, areas where it could have proven of great advantage (Silverstein, 2012). It seemed the public were more accepting in 1796 when Edward Jenner demonstrated a new procedure called vaccination. This was essentially the same concept as variolation, but less dangerous as it involved infecting patients with the mild disease cowpox in order to protect against the deadly smallpox (Murphy, 2011). Concepts of disease and immunity were evolving, and in 1858 a great leap was made when Rudolph Virchow dared to challenge the humoral theory. He suggested instead, that illness could be a result of faulty cells (Silverstein, 2012).

1.1.2 The beginning of Immunology as a discipline

Immunology as a discipline began in the late 1870s, following acceptance of the germ theory of disease (Silverstein, 2012). It was Louis Pasteur and Robert Koch who finally proved that disease was not an affliction by god or an imbalance of humors, but was instead caused by tiny unseen microorganisms (Silverstein, 2012). Far from collaborators, however, Pasteur and Koch had an open rivalry and, throughout their working lives, would treat one another's ideas with great distain (Davis, 2013; Silverstein, 2012).

In 1890 came the discovery by Emil von Behring and Kitasato Shibasaburo that immunisation caused the appearance of a soluble substance in the blood that was able to neutralise toxins (Silverstein, 2012). These "antitoxins" were to later become known as antibodies. Their presence in the body fluids seemed to conform to the ancient Greek system of the humors and consequently, the study of antibodies became known as that of humoral immunity. Around the same time, Ilya (Elie) Metchnikoff was working on his cellular theory of immunity. In 1891 Metchnikoff published his "Lectures on the Comparative Pathology of Inflammation". In these he explained his revolutionary ideas regarding the phagocytosis of pathogens by immune cells and the protective role of inflammation (Silverstein, 2012).

From this point forward, immunologists became divided into those who believed that humoral mechanisms best explained immunity and those who championed the cell as the body's guardian. The humoralists came mostly from Germany and were led by Koch in Berlin, while the cellularists were from France and followed Metchnikoff, who was working at the Pasteur Institute in Paris (Davis, 2013; Silverstein, 2012).

1.1.3 Early theories of antibody formation – Immunochemistry

A turning point was reached in 1897 when Paul Ehrlich published his paper describing the "side-chain theory" of antibody formation. He postulated that specific receptors for toxin molecules exist on the surface of certain cells. This was the first natural selection theory of antibody formation (Silverstein, 2012). In a 1903 paper, Almroth Wright described how humoral antibodies could opsonise target pathogens, making them vulnerable to phagocytosis by macrophages. Sadly, his results proved unreproducible at the time and were largely forgotten (Silverstein, 2012). The cellular theory of immunity became unfashionable for many years to come. Consequently, the first half of the 20th century saw a decline in cellular research and an almost complete preoccupation with the antibody. Antibodies, after all, were much easier to work with than cells.

Scientists became convinced that understanding the chemistry and thermodynamics of antibodies was the way forward. A new branch of immunology was born, that of immunochemistry (Silverstein, 2012). Over the next fifty years several incorrect theories of antibody formation were proposed. Many, including Linus Pauling, believed in the "instruction" or "template" theories – the idea that the antibody moulded itself around the antigen (Pauling and Delbrück, 1940; Silverstein, 2012). Others believed that the antibody was fashioned from part of the antigen molecule itself (Silverstein, 2012). Disagreeing with the instructional theories, Niels Jerne suggested his "natural-selection theory" in which all manner of different "natural" antibodies pre-existed in healthy blood, waiting for foreign molecules to which they could bind (Jerne,

1955). Although this theory incorporated many of Ehrlich's ideas, Jerne did not refer to him in his 1955 paper (Jerne, 1955). Frank Macfarlane Burnet liked Jerne's hypothesis. In 1957 Burnet published "A modification of Jerne's theory of antibody production using the concept of clonal selection" (Burnet, 1976). In it he described how, upon seeing a molecule to which its antibodies could bind, the cell could proliferate and form many clones. One cell would make only one shape of antibody. Importantly, Burnet brought the focus away from the antibody itself and towards the cell making it.

1.1.4 A return to Immunobiology

From the second half of the 20th century onwards immunology became, once again, more medically and biologically orientated. Peter Medawar's experiences with badly burned patients during the Second World War had forced him to consider the immunology of transplantation (Davis, 2013). Allografts were rejected whilst autografts were not. Suddenly the "self-marker" hypothesis, as first described by Burnet and Frank Fenner in 1949, became entirely relevant. This was the idea that the immune system's sole purpose was the discrimination of "self" from "non-self" (Davis, 2013). Medawar soon realised that the distinction between self and non-self was learned. Immunological tolerance, it seemed, was something to be acquired.

However, to the immunologist's surprise, it was discovered that antibodies were not responsible for the rejection of transplanted tissues. In the 1950s and 1960s a number of independent groups demonstrated that graft rejection was instead due to the differences between the donor and recipient of certain groups of "compatibility genes" – those belonging to the major histocompatibility complex (MHC) (Davis, 2013).

1.1.5 The discovery of T cells

Removal of the avian bursa of Fabricius (shown in the 1970s to be the equivalent of the mammalian bone marrow) resulted in severely reduced

antibody formation (Glick et al., 1956; Kincade and Kelsoe, 2003). A similar experiment, performed in 1961 and involving removal of the thymus from neonatal mice, proved to be of immense importance (Miller, 1961). Remarkably, the thymectomized mice were unable to reject skin grafts (Miller, 1961). The thymus, previously unappreciated, became an organ of great interest.

Burnet was among the first to recognise the true significance of the thymus. In a lecture that he gave in London in 1962, he suggested that the thymus had an important role to play in the prevention of self-reactivity (Davis, 2013). It soon became understood that there were two types of lymphocytes, B (bursa/bone marrow) cells and T (thymus) cells. In 1969 Avrion Mitchson showed that T cells cooperate with B cells in the production of antibodies (Silverstein, 2012). A short time later, in 1975, Rolf Zinkernagal and Peter Doherty noted that T cells recognise antigen only when it is presented as an amino acid sequence bound to the surface of an MHC molecule (Silverstein, 2012). In this way, the T cell recognises both self and non-self. Suddenly the enigma surrounding transplant rejection was solved - the recipient's T cells could recognise the donor's MHC molecules as foreign and mount an attack.

In the 1970s immunologists started to classify T cells into different subsets, depending on their function and surface markers. Helper and cytotoxic T cells were described, as were T cells with a suppressor (regulatory) function (Gershon and Kondo, 1970; Silverstein, 2012). Today T cell immunology is still in its relative infancy. The establishment of the cluster of differentiation (CD) nomenclature in 1984 was particularly useful to T cell immunologists (Zola et al., 2005). So too was the development of fluorescent antibodies and the technique of fluorescence activated cell sorting (FACS) (Herzenberg et al., 2002). It seems the era of the T cell is still very much upon us. Quite ironic really, that it should be the monoclonal antibody that is proving of limitless aid to today's cellular immunologist!

1.2 Our current understanding of the immune system

Most infectious organisms are prevented from entering the body by the protective epithelial layers of the skin and mucous membranes (Murphy, 2011). If however, these barriers are breached then the immune system is activated to both contain and clear the infection. The immune system is made up of cells and molecules. Essentially, it is comprised of two branches: the innate ("natural") immune system and the adaptive ("acquired") immune system (Fearon and Locksley, 1996). Whilst all multicellular organisms possess some form of innate immunity to protect them from invaders, the adaptive immune system is found only within vertebrates (Hedrick, 2004). The vertebrate immune system must be able to: recognise infection; mount a response via a variety of effector mechanisms; regulate itself to prevent damage to the host; and finally, provide immunological memory, so that a second response to the same infection is both more rapid and of a greater magnitude (Murphy, 2011).

In vertebrates, all immune system cells are derived from pluripotent haematopoietic stem cells situated in the bone marrow. Such cells give rise to the myeloid and lymphoid lineages of leukocytes (Murphy, 2011). Common myeloid progenitors produce dendritic cells (DCs), macrophages, mast cells, and granulocytes (neutrophils, eosinophils, and basophils). Macrophages are the mature, tissue resident form of monocytes. Common lymphoid progenitors produce B and T lymphocytes and natural killer (NK) cells (Murphy, 2011). Although most commonly of myeloid lineage, dendritic cells can also arise from lymphoid progenitors (Wu and Liu, 2007). The B and T cells are responsible for the adaptive immune response and hence for the acquisition of immunological memory. All other cell types are classified as belonging to the innate immune system, although their actions frequently aid the adaptive response (Murphy, 2011). Macrophages, granulocytes, and dendritic cells can all internalise pathogens by the process of phagocytosis (Aderem and Underhill, 1999).

Cells of the innate immune system possess invariant cell surface and intracellular receptors, capable of recognising constituents common to many

pathogens. Many secreted molecules of innate immunity are also able to recognise repeating microbial structures (Quah and Parish, 2001). In contrast, the cell surface receptors of B and T cells are of unlimited variety and are highly specific for their cognate antigens (Hedrick, 2004).

In many cases, pathogen clearance requires only the innate immune system. If however, innate mechanisms prove insufficient, then the adaptive immune system is activated. Some components of the innate response lie resident in healthy tissues and are available for immediate use upon infection. Other components are induced in response to infection and appear over a matter of hours (Murphy, 2011). The innate response works to contain the infection for several days, until the adaptive response can be initiated. Crucially, the adaptive immune system is reliant on the innate immune system for activation (Murphy, 2011).

1.2.1 The early stages of an innate immune response

Upon infection of the tissue, an innate immune response is immediately activated. Antimicrobial proteins are present within saliva and tears and can also be secreted by epithelial cells and phagocytes. They include defensins, cathelicidins, histatins, and lysozyme (Cash et al., 2006; De Smet and Contreras, 2005). Such antimicrobial proteins can directly attack the cell walls of bacteria, the cell membranes of bacteria and fungi, and the envelopes of some viruses (De Smet and Contreras, 2005). The complement system of plasma proteins is rapidly activated upon infection. Complement can bind directly to pathogen surfaces or it can bind to proteins (e.g. C-reactive protein, mannose-binding lectin, ficolins, and antibodies) that have previously bound pathogen (Gros et al., 2008). Complement binds particularly well to natural antibodies of the class IgM, which circulate in the absence of infection (Heyman et al., 1988; Rutemark et al., 2012). Complement can either directly lyse pathogens or it can opsonise them, thus facilitating their uptake by phagocytes such as macrophages (Gros et al., 2008).

Macrophages are tissue resident cells and are of immense importance in the early stages of the immune response (Quah and Parish, 2001). In addition to complement receptors, macrophages also possess a huge variety of receptors that can bind to the carbohydrates, lipids, and lipoproteins of pathogen surfaces (Aderem and Underhill, 1999). Such receptors are termed pattern recognition receptors (PRRs) and the conserved structures they recognise are called pathogen associated molecular patterns (PAMPs). Macrophage PRRs include Toll-like receptors (TLRs), NOD-like receptors (NLRs), and RIG-I like receptors (RLRs) (Thompson et al., 2011). They recognise bacterial, viral, and fungal constituents. Upon activation of complement receptors or the cell surface TLRs, macrophages are triggered to phagocytose the pathogen (Quah and Parish, 2001).

The macrophage can then be activated by stimulation of cell surface G-protein coupled receptors (e.g. the fMet-Leu-Phe receptor) (Lattin et al., 2007). This leads to the subsequent killing of phagocytosed pathogens. The G-protein coupled receptors can recognise bacterial components, but can also respond to the anaphylatoxins (e.g. C5a) produced by activation of the complement cascade and to chemokines (Lattin et al., 2007). Upon activation of the macrophage, intracellular vesicles containing the phagocytosed pathogens (phagosomes) fuse with lysosomes and generate phagolysosomes (Slauch, 2011). These are highly acidic vesicles that contain lysosomal hydrolases as well as reactive oxygen and nitrogen species (Slauch, 2011). The pathogen is rapidly degraded within the phagolysosome (Slauch, 2011).

1.2.2 The induced stages of an innate immune response

Within hours of the infection occurring, many innate effector mechanisms will have been induced. This induced phase of innate immunity can last over several days and is important for containing the infection until the adaptive immune response is fully activated. In response to stimulation of their TLRs, NLRs, or RLRs, macrophages can activate the NF-κB signalling pathway (Quah and Parish, 2001). This results in the production of cytokines and chemokines.

Cytokines and chemokines are small proteins that affect the behaviour of surrounding cells and are important for the initiation of an inflammatory response (Borish and Steinke, 2003). Together with anaphylatoxins, cytokines can cause vasodilation and an increase in the permeability of the blood vessels (Markiewski and Lambris, 2007).

Cytokines activate endothelial cells and increase their expression of selectins, a group of adhesion molecules (Granger and Senchenkova, 2010). The cytokine, tumour necrosis factor (TNF), is a particularly good inducer of P-selectin and E-selectin (Bullard et al., 1996). The selectins of endothelial cells bind weakly to sulphated sialyl-Lewis^x carbohydrates that are present on the surface of circulating neutrophils and monocytes (Murphy, 2011). By binding to selectins, leukocytes are able to adhere to the blood vessel wall and roll slowly along it. Activated endothelial cells also express intercellular adhesion molecules (ICAMs). The ICAMs bind to integrins present on the surface of leukocytes. When chemokines bind to the chemokine receptors of leukocytes, a conformational change is induced within the integrins (Alon and Feigelson, 2002). This enables tight binding between ICAMs and the integrins. In response to this, the leukocyte then stops rolling and is instead bound tightly to the wall of the blood vessel. The leukocyte may then squeeze between the tight junctions of adjacent endothelial cells and pass through the underlying basement membrane in a process called diapedesis (Johnston and Butcher, 2002). Upon completion of diapedesis, leukocytes migrate deeper into the tissue, following the chemokine gradient towards the site of infection (Murphy, 2011). The whole process of leukocyte movement from the blood vessels into the tissues is termed extravasation (Alon and Feigelson, 2002).

In addition to aiding cell migration, vasodilation and increased vascular permeability also increase the flow of fluid from the bloodstream into the tissues. In this way, plasma proteins, including complement and antibodies, are brought to the site of infection and the increased fluid pressure in the tissues also encourages lymphatic drainage to the lymph nodes (Murphy, 2011). Movement of cells and fluid from the bloodstream and into the tissues is

accompanied by heat, redness, swelling, and pain – the clinical markers of an inflammatory response (Murphy, 2011).

TNF stimulates endothelial cells to release blood-clotting factors (Granger and Senchenkova, 2010). By preventing blood flow in the small, downstream blood vessels, the infection is kept localised and prevented from disseminating throughout the body. If the infection does reach the bloodstream then sepsis may occur. This leads to the activation of macrophages in the liver and spleen, which release huge quantities of TNF, causing septic shock (Dellinger, 2003). The resulting systemic vasodilation plus increased vascular permeability and disseminated intravascular coagulation are very dangerous to the host and frequently prove fatal. (Dellinger, 2003)

Following infection, neutrophils are the first cells to extravasate into the tissues. They do so in great numbers, following the CXCL8 chemokine gradient (Kolaczkowska and Kubes, 2013). Monocytes enter the tissues slightly later, in response to the chemokine CCL2 (Kolaczkowska and Kubes, 2013). Neutrophils are highly phagocytic. Like macrophages, they possess numerous receptors that enable phagocytosis and cell activation (Kolaczkowska and Kubes, 2013). Neutrophils contain primary and secondary granules that fuse with the phagosome. This subjects the pathogen to killing by antimicrobial proteins, enzymes, and the respiratory burst. Neutrophils are short-lived, dying after use of their granules (Kolaczkowska and Kubes, 2013).

Many immature dendritic cells lie resident within the tissues, surveying for the presence of infection. However, upon activation of the inflammatory response, dendritic cells are recruited to the tissues in larger numbers (Shortman and Liu, 2002). Monocytes extravasating from the bloodstream usually turn into macrophages in response to the cytokine, macrophage colony-stimulating factor (M-CSF). However, upon exposure to both granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-4, monocytes will instead enter the tissues as dendritic cells (Palucka et al., 1998; Shortman and Liu, 2002). Immature dendritic cells possess many PRRs and complement receptors and are capable of phagocytosing pathogens (Lim and Gleeson, 2011). In addition,

they can continually take up extracelluar fluid from their surroundings by the process of macropinocytosis, which does not require receptor stimulation (Lim and Gleeson, 2011). Uptake of pathogens allows the dendritic cell to mature. NF-κB signalling occurs downstream of TLRs and NLRs (Quah and Parish, 2001). This stimulates the production of cytokines and chemokines and also leads to increased expression of the co-stimulatory molecules CD80 (B7.1) and CD86 (B7.2) on the dendritic cell surface (Shortman and Liu, 2002).

Both macrophages and dendritic cells secrete large amounts of the cytokines IL-1β, IL-6, IL-12, and TNF and the chemokine CXCL8 (Murphy, 2011). IL-1β, IL-6, and TNF are important activators of the acute-phase response (Murphy, 2011). They stimulate hepatocytes to release acute-phase proteins (e.g. C-reactive protein), which assist in the opsonisation of pathogens (Borish and Steinke, 2003). TNF can also stimulate mature dendritic cells to enter the lymphatic vessels, which carry them to the draining lymph nodes (Förster et al., 2012). It is these dendritic cells that provide a crucial bridge between the innate and adaptive branches of the immune system (Shortman and Liu, 2002).

1.2.3 The adaptive immune system

If an infection cannot be controlled by the innate immune system alone, then the adaptive immune system is activated. An adaptive immune response is initiated in the secondary lymphoid organs. Mature dendritic cells travel from the site of infection and enter the draining lymph nodes, or other nearby lymphoid tissues (Shortman and Liu, 2002). They present the pathogen on their cell surface in the form of peptide antigens bound to molecules of the major histocompatibility complex (MHC) (Shortman and Liu, 2002). In the lymphoid organ, the dendritic cells will come into contact with large numbers of naïve T cells. If a T cell possesses a cell surface receptor (T cell receptor, TCR) with the correct specificity for the peptide:MHC complexes presented by the dendritic cell, then the T cell may become activated. The T cell can then undergo clonal expansion and differentiate into one of several types of effector T cell.

The cytokines produced during the innate stages of the immune response help to determine the types of effector T cell that are produced (Murphy, 2011). The effector T cells travel from the lymphoid organs and eventually into the bloodstream. They circulate in the blood until they encounter endothelial activation at the site of infection. They can then extravasate into the infected tissue (Murphy, 2011). T cells may either kill infected cells directly or may activate other cells of the immune system.

During an infection, complement coated antigens can be transported into the secondary lymphoid organs where they can be presented, in their native form, on the surface of follicular dendritic cells (FDCs, cells of nonhaematopoietic origin) and on specialised macrophages (Batista and Harwood, 2009). Naïve B cells enter the lymphoid organs, where they reside initially in the B cell zone, also called the primary lymphoid follicle (Batista and Harwood, 2009). Upon sensing an antigen (on the FDCs or macrophages) to which their B cell receptor (BCR) can bind, the B cells begin to express the chemokine receptor CCR7 (Okada et al., 2005). This enables the B cell to move, within the secondary lymphoid organ, to the edge of the T cell zone (Okada et al., 2005).

During T cell differentiation, a subset of effector T cells, called T follicular helper (Tfh) cells, develop. These Tfh cells remain in the secondary lymphoid organs, where they may recognise peptide antigens as displayed on MHC class II molecules on the surface of the B cell (Ma et al., 2012). If so, then the Tfh cell can activate the B cell. Activated B cells are then able to undergo clonal expansion and differentiation, and they form a primary focus as they do so (Murphy, 2011). After several days, B cells differentiate into plasma cells. Some plasma cells stay in the lymphoid organs, while others travel to the bone marrow (Radbruch et al., 2006). The plasma cells will secrete large quantities of antibody, which enter into the bloodstream and eventually, into the site of infection. Antibodies may help opsonise the pathogen; can activate cells such as mast cells, eosinophils, and basophils; and can initiate the classical complement cascade (Murphy, 2011).

The cell-mediated response provided by activated T cells, and the humoral response provided by activated B cells work in harmony with the innate immune system to clear the infection (**Table 1.1**). Immunological memory is also acquired during the adaptive stage of the primary immune response. This involves the production of long-lived memory T and B cells (Farber et al., 2013; Radbruch et al., 2006). Immunological memory ensures that a secondary infection with the same pathogen can be cleared more rapidly than the first or, in some cases, that reinfection is entirely prevented.

Table 1.1 Interactions between the innate and adaptive branches of the immune system

An immune response takes time to develop and certain immune system components or cell types will, by their very nature, become active at earlier or later stages during the response. However, this does not mean that their actions are confined to a short timeframe. Nor does it mean that there is a true dichotomy between the innate and adaptive branches of the immune system. As this table shows, the different components and cells of the immune system are in constant interaction with one another. For example, T cells form part of the adaptive immune system and are not activated until around 4 days after the initial infection. Yet a T cell's actions and the cytokines it secretes can have profound influences on cells that were recruited much earlier during the immune response. This table shows when the different components of the immune system are first activated during the immune response. Activation may occur during the early innate (0-4 hours, green boxes), induced innate (4-96 hours, blue boxes), or adaptive (>96 hours, purple boxes) phases of the immune response (Murphy, 2011). Certain components, such as cytokines and chemokines, play a crucial role at all stages of the immune response (orange boxes). The table also shows the mechanisms of action of the different immune system components and gives examples of how the different components may aid one another.

PRR = pattern recognition receptor. PAMP = Pathogen associated molecular pattern. MHC = major histocompatibility complex.

Table constructed by myself. Inspiration taken from (Murphy, 2011).

Key

Early innate response (0-4 hours)

Induced innate response (4-96 hours)

Adaptive response (>96 hours)

Throughout the immune response

Component of the immune system	Mechanism of action	Aided by
Antimicrobial proteins (e.g. cathelicidins, histatins, lysozyme) found in tears & saliva & secreted by epithelial cells	Attack cell walls of bacteria, cell membranes of bacteria & fungi, & envelopes of some viruses.	Cytokines & chemokines (activate epithelial cells)
Complement system of plasma proteins	Binds pathogen surfaces directly leading to lysis or opsonisation. Binds C-reactive protein, mannose binding lectin, IgM Ab.	B cells (IgM production)
Macrophages	Their PRRs bind PAMPs of pathogen surfaces. Phagocytose the pathogen. Stimulation of a macrophage's G-protein coupled receptors leads to killing of phagocytosed pathogens.	Complement cascade (produces anaphylatoxins, which stimulate G-protein coupled receptors)
		T cells (helper T cells activate macrophages)
Mast cells	Secrete inflammatory proteins, causing smooth muscle contraction.	B cells (IgE produced by B cells binds the surface of mast cells and is cross-linked by antigen, thus activating the mast cell)
Cytokines & chemokines	Cause vasodilation & increased blood vessel permeability. Activate endothelial cells. Enable extravastion of leukocytes from the blood vessels into the	Many immune cell types, both innate & adaptive, secrete large quantities of

	tissue. Enable the differentiation or activation of immune cells.	cytokines & chemokines
Neutrophils	Phagocytose pathogen & kill it through use of primary & secondary granules.	Macrophages (produce CXCL8, enabling neutrophil recruitment into the tissue)
Monocytes	Recruited to tissues & become macrophages (see above entry for macrophages).	Multiple cell types produce CCL2, which recruits monocytes into the tissue
Dendritic cells (DCs)	Their PRRs bind PAMPs on pathogen surfaces. Phagocytose pathogens & present them on MHC complexes on their cell surface. Produce many cytokines & chemokines.	T cells (produce IL-4: helping to turn infiltrating monocytes into DCs instead of macrophages)
		Complement cascade (opsonises pathogens)
Natural killer (NK) cells	Release cytotoxic granules containing perforin & granzyme, leading to lysis of infected cells. Produce IFN-γ.	B cells (antibody dependent cellular cytoxicity, ADCC, occurs when an Ab coated target cell is recognised and lysed by an NK cell)
		DCs (plasmacytoid DCs produce much IFN-α & IFN-β, which activates NK cells)
		Macrophages (release IL-12 & IL-18, which activate NK cells)
Eosinophils	Secrete inflammatory proteins, causing smooth muscle contraction.	B cells (IgE produced by B cells binds the surface of eosinophils and is cross-linked by antigen, thus activating the eosinophil)

		Mast cells (secrete cytokines that cause eosinophil activation)
Basophils	Secrete inflammatory proteins, causing smooth muscle contraction.	B cells (IgE produced by B cells binds the surface of basophils and is cross-linked by antigen, thus activating the basophil)
Acute phase proteins (e.g. fibrinogen, C-reactive protein), which are released by hepatocytes in response to IL-1, IL-6, IL-8, & TNF	Coagulation of blood vessels. Opsonisation of pathogens.	Macrophages (secrete large quantities of IL-1, IL-6, IL-8, & TNF)
		DCs (secrete large quantities of IL-1, IL-6, IL-8, & TNF)
		Mast cells (major TNF producers)
T cells	Differentiate into one of several different types of effector T cell. Can either kill infected cells directly or can activate other cells of the immune system.	DCs (present peptide:MHC to the T cell to activate it. In addition, are major producers of cytokines necessary for T cell differentiation)
		Macrophages (major producers of cytokines necessary for T cell differentiation)
		NK cells (produce IFN-γ, which enables production of Th1 cells)
B cells	Produce antibodies, which can: opsonise pathogens; neutralise toxins; activate the complement cascade; and activate mast cells, NK cells, eosinophils, & basophils.	T cells (Tfh cells help to activate B cells)

1.3 T cell development

1.3.1 The thymus

T cell progenitors arise in the bone marrow, however they must travel to the thymus in order to complete their maturation. The thymus is located in the thorax or neck of all vertebrates (Pearse, 2006). In humans and mice, it is a bi-lobed organ, located behind the sternum and in front of the heart. The thymus is at its largest early in life, with thymic involution occurring from adolescence onwards (Pearse, 2006). Essentially, the thymus is composed of an outer cortex and an inner medulla (**Schematic 1.1**). Immature thymocytes and low numbers of macrophages reside in the cortex, whilst more mature thymocytes and large numbers of both macrophages and dendritic cells are present within the medulla (Pearse, 2006). Epithelial stromal cells within the thymus are important for the provision of both survival and differentiation signals to the developing T cells (Takahama, 2006).

The thymus of mice has been shown to contain huge numbers of thymocytes (approximately 10^8 to 2×10^8), however, each day, only a small percentage of these migrate into the periphery (Pozzesi et al., 2014). In fact, an estimated 98% of developing T cells will undergo apoptosis within the thymus (Pozzesi et al., 2014). It was for this very reason that early immunologists wrongly dismissed the thymus as having no real function, other than as a graveyard for dead and dying T cells (Miller, 2014).

"*We shall come to regard the presence of lymphocytes in the thymus as an evolutionary accident of no very great significance*"

> Peter Medawar, 1963
> (Medawar's comments were in response to an earlier publication by Miller, 1961; as quoted in Miller, 2014)

Today we understand the true importance of the thymus in the production of T lymphocytes and the acquisition of immunological tolerance.

1.3.2 The double negative stage of development

T cell progenitors travel from the bone marrow to the thymus via the bloodstream. They enter the cortico-medullary junction of the thymus through the high endothelial venules (HEVs). Once in the thymus, the progenitors receive signalling through their Notch1 receptors, thereby triggering their commitment to the T cell lineage (Borowski et al., 2002). Of note, the conditional deletion of Notch1 in bone marrow precursors results in the appearance of B cells in the murine thymus (Wilson et al., 2001). Signalling via the Kit and IL-7R cytokine receptors is also important during early thymocyte development (Borowski et al., 2002). Their ligands (stem cell factor and IL-7, respectively) are both produced by thymic stromal cells (Borowski et al., 2002).

Upon entering the thymus, cells lack expression of the TCR and the CD4 and CD8 co-receptors. Due to their absence of both CD4 and CD8, these immature thymocytes are referred to as double negative (DN). Around 5% of thymocytes are classified as DN (Ceredig and Rolink, 2002). Cells within the DN stage of development can be further characterised into developmentally sequential subsets (DN1-4) according to their expression of CD44 (an adhesion molecule) and CD25 (the IL-2R α-chain). As defined by Godfrey et al.: DN1 thymocytes are $CD44^{+}CD25^{-}$, DN2 thymocytes are $CD44^{+}CD25^{+}$, DN3 thymocytes are $CD44^{-}CD25^{+}$, and DN4 thymocytes are $CD44^{-}CD25^{-}$ (Godfrey et al., 1993). Whilst still in the DN stage, thymocytes migrate from the cortico-medullary junction, through the cortex and into the outermost subcapsular region (Benz et al., 2004). CCR9 seems to be important for this migration, since, in CCR9 deficient mice, DN2 and DN3 thymocytes fail to home to the subcapsular region and are instead distributed across the cortex (Benz et al., 2004).

In order to protect the host from all potential infectious insults, a huge diversity of TCRs is required. However, after it was established that the human genome contains only around 25,000 genes, it became apparent that a separate gene could not possibly encode for each TCR (Silverstein, 2012). From the mid 1970s, Susumu Tonegawa demonstrated that the genes encoding for variable

regions of the BCR could undergo somatic recombination (Hozumi and Tonegawa, 1976). Later this was also shown true for the genes encoding for the variable regions of the TCR (Chien et al., 1984). Although the vast majority of thymocytes that exit the thymus will express an α:β TCR, a small number (an estimated 1-10%) will instead express a γ:δ TCR (Girardi, 2006). Following their successful production, an α-chain will combine with a β-chain to form the α:β TCR, or alternatively, a γ-chain will combine with a δ-chain to form the γ:δ TCR. The TCRβ and TCRδ loci contain variable (V), diversity (D), and joining (J) gene segments, whilst the TCRα and TCRγ loci contain only V and J segments (Girardi, 2006). The somatic recombination of TCR gene segments requires the activity of recombination activating gene 1 (RAG1) and 2 (RAG2) (Schatz and Ji, 2011).

During the DN stage of development, the genes encoding for the TCR begin to rearrange, and it is at this point that the T cell may enter into either the α:β or the γ:δ lineage. At DN1, the TCR genes are still in their germline configuration. During the DN2 stage, the TCR β-chain locus begins to rearrange when D_β combines with J_β (Ceredig and Rolink, 2002). Simultaneously, however, rearrangement also occurs at the γ-chain and δ-chain loci (Ceredig and Rolink, 2002). At DN3, V_β may combine with DJ_β, thus completing rearrangement of the TCR β-chain (Ceredig and Rolink, 2002). TCRβ can then be combined with a pre-Tα (pTα) chain and subsequently expressed as a pre-TCR on the surface of the DN3 thymocyte (Ceredig and Rolink, 2002) **Schematic 1.1**).

Expression of the pre-TCR results in weak activation of Erk (extracellular signal-regulated kinase) (Michie et al., 1999). This is necessary to prevent any further rearrangements occurring at the γ and δ-loci. If however, the γ and δ-loci complete rearrangement before the β-locus, then a γ:δ TCR may be expressed. This causes much stronger Erk signalling, which leads to the activation of the transcription factor Id3 and prevents further rearrangement of the β-locus (Hayes et al., 2005; Kreslavsky et al., 2010). In *Id3*$^{-/-}$ mice, the development of γ:δ T cells is disrupted (Lauritsen et al., 2009). Upon maturation, the γ:δ T cell can exit the thymus and enter the periphery. If thymocytes fail to make either a functional β-chain, or both a functional γ and δ-chain, then they will undergo

apoptosis. In a normal mouse thymus, approximately 20% of DN3 thymocytes will express a γ:δ TCR (Serwold et al., 2007).

Cells that reach the DN4 stage of development will possess a functional β-chain and be committed to the α:β lineage (Ceredig and Rolink, 2002). During DN4, signalling through the pre-TCR allows proliferation and begins production of both CD4 and CD8 (Murphy, 2011). Pre-TCR signalling also prevents further rearrangement of the β-chain locus, thus enabling allelic exclusion. Allelic exclusion is demonstrated in TCRβ transgenic mice, which carry pre-arranged β-chain loci in their germline DNA (Uematsu et al., 1988). Nearly all the T cells produced from these mice will express the same TCR β-chain on their cell surface (Uematsu et al., 1988).

1.3.3 The double positive stage of development

Immediately after passing through the DN4 stage, thymocytes will express cell surface CD8, but lack expression of CD4. These cells are known as immature single-positive thymocytes (ISPs) (Xiong et al., 2011). Upon gaining CD4, the thymocyte progresses into the CD4 CD8 double positive (DP) stage. DP thymocytes are found tightly packed within the inner cortex of the thymus (Dzhagalov and Phee, 2012). At first, DP thymocytes are large in size and proliferate rapidly. They later stop dividing and decrease in size. Whilst in the small DP stage, thymocytes begin to rearrange their TCR α-chain locus (Murphy, 2011). Once a successful rearrangement has been made, an α-chain can be produced and expressed with the β-chain on the surface of the thymocyte (**Schematic 1.1**).

Following α:β TCR expression, rearrangement of the α-chain can continue until the DP thymocyte recognises self-peptide:MHC (Borgulya et al., 1992). In the mouse, α-chain rearrangement is estimated to occur over a 3 to 4 day period (Huesmann et al., 1991). Allelic exclusion does not occur for the TCR α-locus, and any one T cell may theoretically express two different α-chains complexed

to identical β-chains (Malissen et al., 1988; Padovan et al., 1993). In reality, however, it is highly unlikely that both TCRs would be functional.

1.3.3.1 Positive selection

During the small DP stage, thymocytes express only low levels of the α:β TCR on their cell surface (Murphy, 2011). Within the inner cortex and making close contact with the DP thymocytes, are large numbers of cortical thymic epithelial cells (cTECs) (Klein et al., 2014). Such cells are unusual in that they express both MHC class I and MHC class II molecules on their cell surface (Klein et al., 2014). Through interactions with the cTECs, the TCR is checked for self-reactivity. A successful interaction of the TCR with a self-peptide:MHC molecule results in positive selection of the thymocyte, and it is rescued from death (Klein et al., 2014).

Positive selection also requires the interaction of a T cell co-receptor with the invariant portion of an MHC molecule. When the TCR first interacts with self-peptide:MHC it will downregulate both CD4 and CD8. CD4 is then re-expressed (Lundberg et al., 1995). If the thymocyte is interacting with an MHC class II molecule, a strong selection signal is produced, and the thymocyte commits to the CD4 linage. If however, the TCR has bound to MHC class I then a weaker selection signal is induced, and the thymocyte will lose CD4 and instead gain CD8 (Singer et al., 2008).

1.3.3.2 Negative selection

Negative selection can take place in both the cortex and medulla of the thymus and may even be carried out later than the DP stage (McCaughtry et al., 2008). However, it is thought to occur mostly after positive selection and to be induced largely by the interaction of TCR expressing thymocytes with the stromal cells and antigen-presenting cells (APCs) of the thymic medulla (Klein et al., 2014). During negative selection, thymocytes that recognise self-peptide:MHC with too

high an affinity will undergo apoptosis. Crucially, this promotes self-tolerance within the immune system (Klein et al., 2014).

Medullary dendritic cells expressing co-stimulatory molecules are important for the negative selection of thymocytes (Klein et al., 2014). Medullary thymic epithelial cells (mTECs) are also thought to be essential as they can present peripheral self-antigen to the thymocytes (Klein et al., 2014). Expression of the autoimmune regulator (AIRE) gene by the mTECs enables them to express all types of cellular proteins, including ones that are normally restricted to certain tissue types (Klein et al., 2014). AIRE deficient mice exhibit severe autoimmunity, with similarities to the multi-organ autoimmune disease, APECED (autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy), which develops in humans with a defective form of AIRE (Anderson et al., 2002). Negative selection is well demonstrated in mice expressing a TCR specific for the HY (male) antigen. In male mice, DP cells are negatively selected and die by apoptosis, whilst in female mice transgenic T cells populate the periphery (Egawa et al., 2008).

1.3.4 The single positive stage of development

Approximately 1-2% of the DP thymocytes will pass positive and negative selection and mature into single positive (SP) thymocytes expressing either CD4 (CD4SP) or CD8 (CD8SP) (Huesmann et al., 1991). In TCR transgenic mice, where most cells express a selectable TCR, around 20% of DP thymocytes will enter into the SP stage (Huesmann et al., 1991). Of note, CD8 precursors are more susceptible to death than CD4 precursors, and this seems to explain the high CD4:CD8 ratio (approximately 3:1 in a wild-type mouse) present in the thymus (Sinclair et al., 2013). SP thymocytes reside only within the thymic medulla (Dzhagalov and Phee, 2012). During the SP stage of development, cells increase expression of their TCRs. Nearly all CD4SP cells can recognise peptide presented on an MHC class II molecule, whilst nearly all CD8SP cells recognise peptide presented by MHC class I (Singer et al., 2008). A small percentage of CD4SP thymocytes have high expression of CD25 and

CTLA-4 (cytotoxic T-lymphocyte-associated protein 4, an inhibitory receptor for B7 molecules) and also express the transcription factor FoxP3 (Maggi et al., 2005). These cells are known as natural regulatory T (T_{reg}) cells. T_{reg} cells are believed to occur when TCR signalling is stronger than that required to produce a conventional CD4SP cell, but not strong enough to induce negative selection (Jordan et al., 2001).

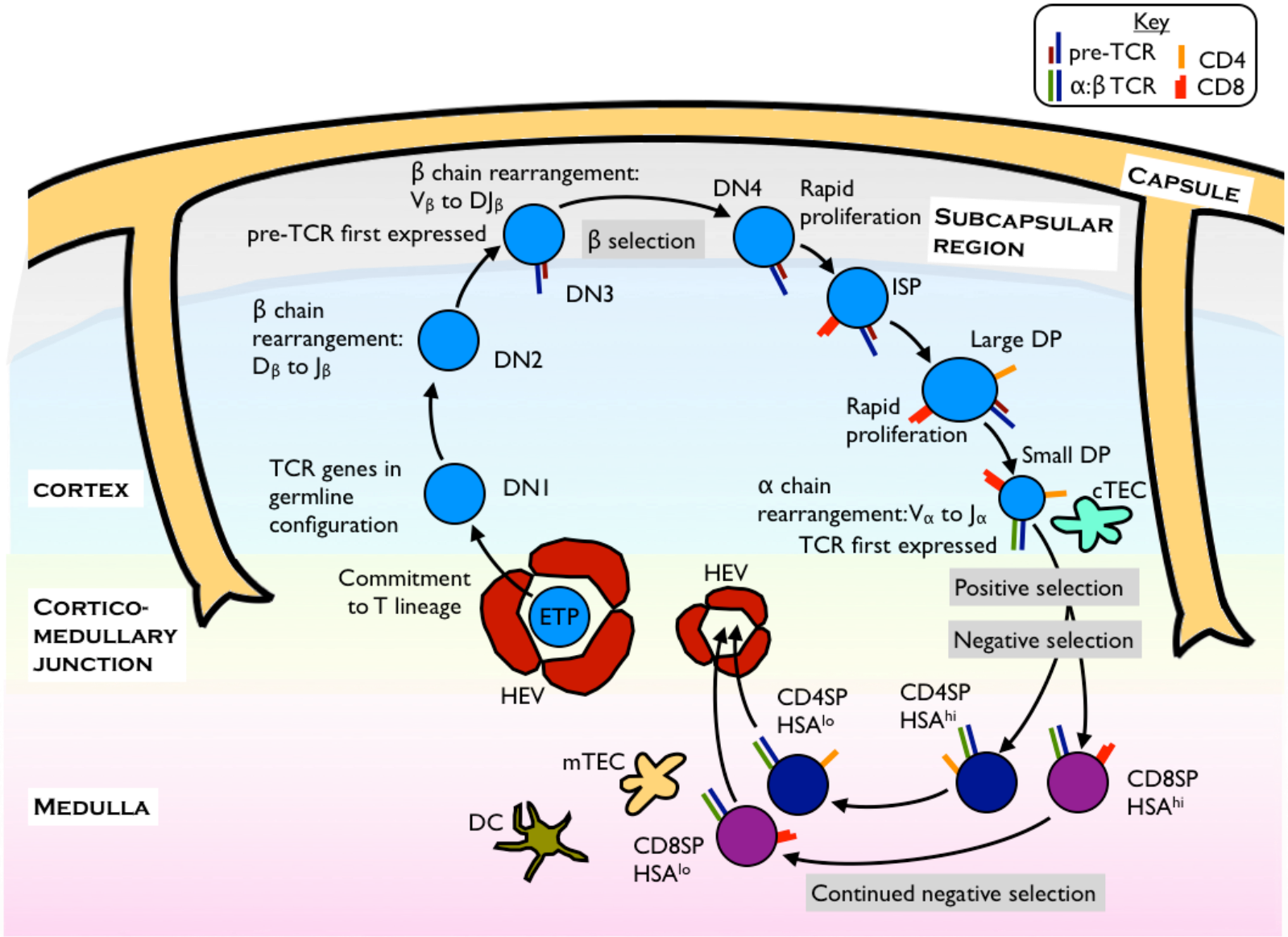
As thymocytes progress through the SP stage of development, they downregulate their expression of the adhesion molecule, heat stable antigen (HSA, CD24) (Tian et al., 2001). Hence, cell surface expression of HSA is a useful marker for distinguishing between immature and mature SP thymocytes. Mature HSA^{lo} SP thymocytes leave the thymus and enter the periphery. In the final stages of maturation, thymocytes begin to express $S1P_1$ (a G-protein coupled receptor) (Takahama, 2006). This enables them to migrate towards sphingosine 1-phosphate (S1P) in the blood and lymph. They also express L-selectin (CD62L), which allows them to home to the peripheral lymphoid organs (Takahama, 2006). Every day 2.6 million CD4SP thymocytes and 0.6 million CD8SP thymocytes are estimated to leave the murine thymus (Sawicka et al., 2014).

Schematic 1.1 T cell maturation in the thymus

Early T cell progenitors (ETPs) enter the cortico-medullary junction of the thymus through the high endothelial venules (HEVs) (Takahama, 2006). Once committed to the T cell lineage, the cells, due to their lack of both CD4 and CD8 surface markers, are referred to as double negative (DN). As the DN cells progress through DN1-DN4, they rearrange their TCR β-chain locus (Ceredig and Rolink, 2002) and migrate from the cortico-medullary junction, through the cortex, and towards the subcapsular region (Benz et al., 2004). DN3 thymocytes that express a pre-TCR on their cell surface may pass through the β selection checkpoint. After progressing through DN4, thymocytes begin to express cell surface CD8, but lack CD4. These cells are referred to as immature single positives (ISPs) (Xiong et al., 2011). Once thymocytes gain CD4 they enter the double positive (DP) stage of development. DP thymocytes are tightly packed within the cortex of the thymus (Dzhagalov and Phee, 2012). At first, they are large in size and undergo rapid proliferation, later they become smaller and begin rearrangement of their TCR α-chain locus (Murphy, 2011). Small DP thymocytes may express the α:β TCR on their cell surface. In the inner cortex, cortical thymic epithelial cells (cTECs) make close contact with the small DP thymocytes and check the α:β TCRs for self-reactivity. A successful interaction between self-peptide:MHC on the cTECs and the α:β TCR on the DP cell results in positive selection of the thymocyte (Klein et al., 2014). During positive selection, the thymocyte will also commit to either the CD4 or CD8 lineage. Successful interaction with MHC class II results in commitment to the CD4 lineage and the production of a CD4 single positive (SP) thymocyte. Alternatively, interaction with MHC class I allows the thymocyte to develop into a CD8SP cell. Negative selection may take place in the cortex or medulla of the thymus (McCaughtry et al., 2008). Stromal cells and antigen presenting cells within the thymus may interact with the α:β TCR expressing thymocytes. If this interaction is too strong then negative selection will occur and the thymocyte will die by apoptosis (Klein et al., 2014). As thymocytes progress through the SP stage of development, they downregulate their expression of heat stable antigen (HSA) (Tian et al., 2001). The mature HSA^{lo} thymocytes leave the thymus and enter the periphery through the HEVs (Takahama, 2006).

mTEC = medullary thymic epithelial cell. DC = dendritic cell.

Schematic drawn by myself. Schematic partially inspired by (Takahama, 2006).


Key
pre-TCR
α:β TCR
CD4
CD8
CAPSULE
SUBCAPSULAR REGION
CORTEX
CORTICO-MEDULLARY JUNCTION
MEDULLA
β chain rearrangement: Vβ to DJβ
pre-TCR first expressed
β selection
DN4
Rapid proliferation
ISP
Large DP
Rapid proliferation
Small DP
α chain rearrangement: Vα to Jα
TCR first expressed
cTEC
Positive selection
Negative selection
DN3
β chain rearrangement: Dβ to Jβ
DN2
TCR genes in germline configuration
DN1
Commitment to T lineage
ETP
HEV
HEV
CD4SP HSAlo
CD4SP HSAhi
CD8SP HSAhi
mTEC
DC
CD8SP HSAlo
Continued negative selection

1.4 The peripheral T cell compartment

1.4.1 T cell activation

After leaving the thymus, mature T cells enter the bloodstream. Before they have encountered their specific antigen, these cells are referred to as naïve. They may recirculate between the blood and secondary (peripheral) lymphoid organs until they either meet their target antigen or die (Cose, 2007). The secondary lymphoid organs are highly organised structures, with B cells and T cells residing in distinct compartments. They include the lymph nodes and spleen, as well as discrete areas within mucosal linings (including the linings of the gut, the nasal and respiratory tracts, and the urogenital tract) (Murphy, 2011).

Stromal cells, present within the T cells zones of the secondary lymphoid organs, secrete the chemokine SLC (secondary lymphoid chemokine, CCL21) (Förster et al., 2012). This enables dendritic cells, expressing the SLC receptor, CCR7, to enter into the T cell zones (Caux et al., 2000). The dendritic cells express more SLC and can also express another chemokine, CCL19 (MIP-3β), which is also a ligand for CCR7 (Caux et al., 2000). T cells expressing CCR7 can then follow the SLC/CCL19 chemokine gradient (Förster et al., 2012). CCR7 deficient mice exhibit an accumulation of T cells in the skin and a decrease in T cell numbers in the lymph nodes (Sumaria et al., 2011). The T cells enter the lymph nodes through the HEVs by the process of extravasation. The initial slow rolling of the T cell along the HEV wall is mediated by L-selectin on the T cell binding to addressins, such as CD34, on the endothelial cells (Puri et al., 1995).

Within the secondary lymphoid organ, the T cell is well placed to make contact with dendritic cells. Naïve T cells interact with the surfaces of dendritic cells, browsing them for the presence of peptide:MHC to which they can bind (Montoya et al., 2002). Respectively, LFA-1 and CD2 on the T cell can bind ICAM-1/2 and CD58 on the DC (Montoya et al., 2002). The binding between the

DC and the T cell will be weak at first. However, if the T cell recognises peptide:MHC complexes on the DC, then the subsequent TCR signalling causes a conformational change in LFA-1, increasing the affinity with which it binds to the ICAMs (Montoya et al., 2002). If the DC is also able to provide co-stimulation to the T cell, then the T cell is primed (Murphy, 2011).

The initial interaction of the T cell with the peptide:MHC and co-stimulatory molecules of the DC cause the T cell to enter the G_1 phase of the cell cycle (Acuto and Michel, 2003). During T cell priming, TCR signalling activates the NFAT, AP-1 and NF-κB transcription factors, which induce IL-2 transcription (Lin and Weiss, 2001). Co-stimulatory signalling also leads to AP-1 and NF-κB activation, further increasing IL-2 production (Acuto and Michel, 2003). The activated T cell generates the IL-2R α-chain, which combines with the IL-2R β and γ-chains already present at the cell surface. This changes the IL-2R into its high affinity form, which is capable of responding to even low concentrations of IL-2 (Gaffen, 2001). Signalling via this receptor then enables the T cell to progress through the cell cycle (Acuto and Michel, 2003). The T cell proliferates in the lymph node, producing many identical copies of itself by clonal expansion. This occurs over a period of several days, during which an antigen specific CD4 T cell is estimated to divide over 20 times (Gudmundsdottir et al., 1999).

TCR signalling induces expression of CD69 on the activated T cell's surface. This results in the internalisation of $S1P_1$ (Bankovich et al., 2010; Spiegel and Milstien, 2011). Removal of $S1P_1$ from the cell surface traps the proliferating T cells within the secondary lymphoid organ. After a few days, $S1P_1$ is again upregulated and the T cell can leave the lymph node, following the S1P gradient into the lymph and blood (Spiegel and Milstien, 2011).

After 4 or 5 days of proliferating, T cells can differentiate into effector T cells, of which there are several types (Murphy, 2011; **Schematic 1.2**). To carry out their functions, effector cells must recognise peptide:MHC molecules on target cells, but they do not require co-stimulation. Effector cells express higher levels of LFA-1 and CD2 than naïve cells (Murphy, 2011). In addition they lack

expression of L-selection, meaning that they can no longer recirculate between the blood and lymph (Marschner et al., 1999). Instead, they express VLA-4. This enables them to bind to VCAM-1 on the endothelial cells of blood vessels and enter into the tissues at sites of infection (Murphy, 2011). Memory cells may also be generated during the primary immune response and upon the initial encounter of a T cell with its specific antigen. Memory T cells respond far more quickly to their peptide:MHC targets than do naïve T cells.

At any one time, it is estimated that 1 in 10,000 to 1 in 1,000,000 naïve T cells will be specific for any given antigen (Murphy, 2011). This figure is termed the precursor frequency. In mice, the precursor frequency of naïve CD8 T cells specific for a particular MHC restricted epitope of LCMV (lymphocytic choriomeningitis virus) has been estimated to be 1 in 200,000 (Blattman et al., 2002). However, following LCMV infection, an approximate 1000-fold increase in this precursor frequency has been observed (Blattman et al., 2002).

1.4.1.1 Co-stimulation - the CD28 and TNF superfamilies

The B7 molecules expressed on DCs are the most well recognised co-stimulatory molecules and will interact with CD28 on the surface of T cells (Greenwald et al., 2004). However, T cells can express a large variety of co-stimulatory receptors, in addition to CD28. Most of these receptors belong to either the CD28 superfamily or the TNF receptor superfamily (TNFRSF). The receptors ICOS (inducible co-stimulator) and CTLA-4 both belong to the CD28 family (Greenwald et al., 2004). ICOS interacts with ICOSL (B7-H2) on DCs. This interaction is believed to promote the differentiation of CD4 helper T cell subsets (Greenwald et al., 2004). CTLA-4 binds to the B7 molecules more strongly than CD28 and works to inhibit T cell activation (Greenwald et al., 2004). Activated T cells will immediately increase their surface expression of CTLA-4. This enables the DCs to interact preferentially with naïve T cells, as opposed to those T cells that have already been primed (Greenwald et al., 2004).

Signalling induced by the TNF family of cytokines can deliver co-stimulation to the T cell via the TRAF dependent activation of the transcription factor NF-κB (Watts, 2004). CD27, 4-1BBL, and OX40 are members of the TNFRSF and can be expressed on T cells (Watts, 2004). T cells also express the TNF superfamily (TNFSF) cytokine, CD40L in its membrane-bound form (Lesley et al., 2006). CD27 is constitutively expressed on T cells and binds CD27L (CD70) on DCs. CD27 deficient mice fail to generate and maintain a memory T cell response (Hendriks et al., 2000).

OX40 expression is induced following activation of the T cell and is believed to be important in maintaining a T cell response and for the acquisition of immunological memory (Watts, 2004). OX40 deficient mice have reduced CD4 T cell responses to viruses (Kopf et al., 1999). The binding of CD40L to the DC's CD40 and of 4-1BB to 4-1BBL on the DC can induce changes in both the T cell and the DC. The T cell receives appropriate co-stimulation, and the DC further increases its expression of co-stimulatory molecules (Watts, 2004).

DCs are the main APCs. Occasionally, however, macrophages and B cells can also be induced to express co-stimulatory molecules and therefore may act as APCs (Murphy, 2011). In the absence of infection, APCs do not express co-stimulatory molecules. If a naïve T cell recognises peptide:MHC in the absence of co-stimulation, then it may become a regulatory T cell, be deleted, or become anergic (inactivated) (Schwartz, 2003). In this way peripheral tolerance can be induced. Anergy is believed to require blockade of TCR signalling by the expression of E3 ubiquitin ligases such as GRAIL and Cbl-b (Lin and Mak, 2007). *Cbl-b*$^{-/-}$ mice develop spontaneous autoimmunity, with large numbers of activated T and B cells infiltrating many organs (Bachmaier et al., 2000).

1.4.2 T cell differentiation and effector functions

Following activation and proliferation, T cells can differentiate into effector subsets (**Schematic 1.2**). CD8 expressing T cells nearly always become cytotoxic T cells (cytotoxic lymphocytes, CTLs) (Weninger et al., 2002). In

contrast, CD4 expressing cells may differentiate into one of a variety of helper T cell subsets, the most well recognised being Th1, Th2, Th17, and Tfh, or into regulatory T (T_{reg}) cells (Schmitt and Ueno, 2015). The different effector subsets may be defined by the surface markers they express, the stimuli that induce their production, the transcription factors they activate, the cytokines they secrete, and the effects they have on their target cells (**Schematic 1.2**).

Since the TCR is membrane bound, T cells must initiate their effector functions via direct cell:cell contact. Upon recognising peptide:MHC complexes, effector T cells may bind tightly to their targets. An immunological synapse (supramolecular activation complex, SMAC) forms between the T cell and the target cell (Grakoui et al., 1999; Ritter et al., 2013). The TCR, co-receptor, and peptide:MHC complexes are clustered together in the centre of the synapse (central SMAC, cSMAC). Adhesion molecules are present at the outer edge of the synapse (peripheral SMAC, pSMAC) (Grakoui et al., 1999; Ritter et al., 2013). Formation of the immunological synapse triggers a reorganisation of the T cell's cytoskeleton and serves to polarise the T cell so that its effector molecules may be directed towards the target (Ritter et al., 2013; Valitutti et al., 1995).

1.4.2.1 Cytotoxic T cells

CD8 expressing, cytotoxic T cells recognise their specific peptide in the context of MHC class I. Within the endoplasmic reticulum, MHC class I molecules obtain peptides derived from the cell's cytoplasm (Russell and Ley, 2003). For this reason, cytotoxic T cells are able to assess their target cells for the presence of intracytoplasmic pathogens such as viruses. At the site of the immunological synapse, the cytotoxic T cell will release cytotoxic granules containing perforin and granzyme (Russell and Ley, 2003). These induce apoptosis of the target cell and of any intracellular pathogens within it. The cytotoxic T cell rapidly dissociates from the dying cell and moves on to its next target (Russell and Ley, 2003).

1.4.2.2 Helper T cells

CD4 T cells recognise their specific peptide as expressed on an MHC class II molecule. MHC class II molecules bind their peptide antigen within intracellular endosomal vesicles (Murphy, 2011). CD4 T cells are therefore able to assess their target cells for the presence of endocytosed pathogens such as bacteria.

During the innate immune response, DCs and macrophages begin to produce IL-12 and NK cells produce IFN-γ (interferon-γ) (Szabo et al., 2003). Among activated CD4 T cells, IL-12 and IFN-γ can induce the JAK-STAT (janus kinase-signal transducer and activator of transcription) signalling pathway, resulting in the activation of STAT1 and STAT4. STAT1 then activates the transcription factor T-bet (Szabo et al., 2003). T-bet induces the expression of IFN-γ and of the IL-12R, thus contributing to the further differentiation of the T cell (Schmitt and Ueno, 2015). The CD4 T cell eventually becomes committed to the Th1 lineage. Upon recognising its target peptide:MHC, the Th1 cell can secrete large amounts of IFN-γ. Th1 cells are good activators of pathogen-containing macrophages. The IFN-γ produced by the T cell can stimulate the IFN-γ receptor present on the macrophage's surface. In addition, CD40L expressed on the Th1 cell can bind CD40 on the macrophage. Activated macrophages produce large amounts of TNF (Szabo et al., 2003). The TNF can act in an autocrine manner, binding TNFR1 on the macrophage's surface. TNF signalling seems necessary to prevent the Th1 cells from causing apoptosis of the macrophage. Mice deficient in TNFR1 fail to contain *Mycobacterium avium* due to Th1 induced macrophage death (Ehlers et al., 2000).

Eosinophils, basophils and mast cells, employed during the early stages of an immune response, can secrete IL-4 (Paul and Zhu, 2010). When activated in the presence of IL-4, CD4 T cells can signal via STAT6 (Schmitt and Ueno, 2015). STAT6 can activate GATA3, which induces transcription of IL-4 and IL-13 (Paul and Zhu, 2010). The T cell then becomes committed to the Th2 lineage. Th2 cells are important in the killing of extracellular parasites such as

helminths. They can active mast cells and eosinophils and they can induce IgE production by B cells (Paul and Zhu, 2010).

A CD4 T cell that is activated in the absence of IL-4 and IL-12, but in the presence of IL-6 and TGF-β, may differentiate into the Th17 subset (Schmitt and Ueno, 2015). Th17 cells are largely defined by their expression of the transcription factor RORγt, which enables them to produce cytokines of the IL-17 family (Littman and Rudensky, 2010). Th17 cells also produce IL-21 and IL-22. The IL-21 acts in an autocrine manner and causes activation of STAT3 (Littman and Rudensky, 2010). Signalling through the IL-23R seems necessary to keep a T cell committed to the Th17 subset. Th17 cells can induce the expression of IL-8 by epithelial cells (Murphy, 2011). This assists in the recruitment of neutrophils to the site of infection. In this manner, Th17 cells aid the clearance of extracellular bacteria and fungi (Littman and Rudensky, 2010).

Tfh cells provide help to B cells within the follicles of the secondary lymphoid organs (Schmitt and Ueno, 2015). *In vitro* production of Tfh cells has proven difficult, and for that reason we know little about how they may be induced. However, IL-6 and the Bcl6 transcription factor are believed to be necessary for Tfh lineage commitment (Ma et al., 2012). Bcl6 induces transcription of CXCR5, which is needed for retention of Tfh within the B cell follicles. ICOS expressed on the Tfh cells can interact with ICOSL on B cells, thus sustaining interactions between the two cell types (Ma et al., 2012).

T_{reg} cells are important for the suppression of an immune response. Natural T_{reg} cells are produced during thymic development (Maggi et al., 2005). In the presence of correct stimulation, naïve CD4 T cells may differentiate into induced T_{reg} cells. In the absence of IL-6 and other pro-inflammatory cytokines, TGF-β is believed to stimulate the production of induced T_{reg} cells (Schmitt and Ueno, 2015). Both natural and induced T_{reg} cells express the CD4, CD25, and CTLA-4 surface markers and the FoxP3 transcription factor. FoxP3 can interfere with the binding of AP-1 and NFAT to the IL-2 promoter and can prevent the production of IL-2 (Schubert et al., 2001; Wu et al., 2006). CTLA-4 on T_{reg} cells competes with CD28 on naïve T cells for binding to the B7 molecules of DCs

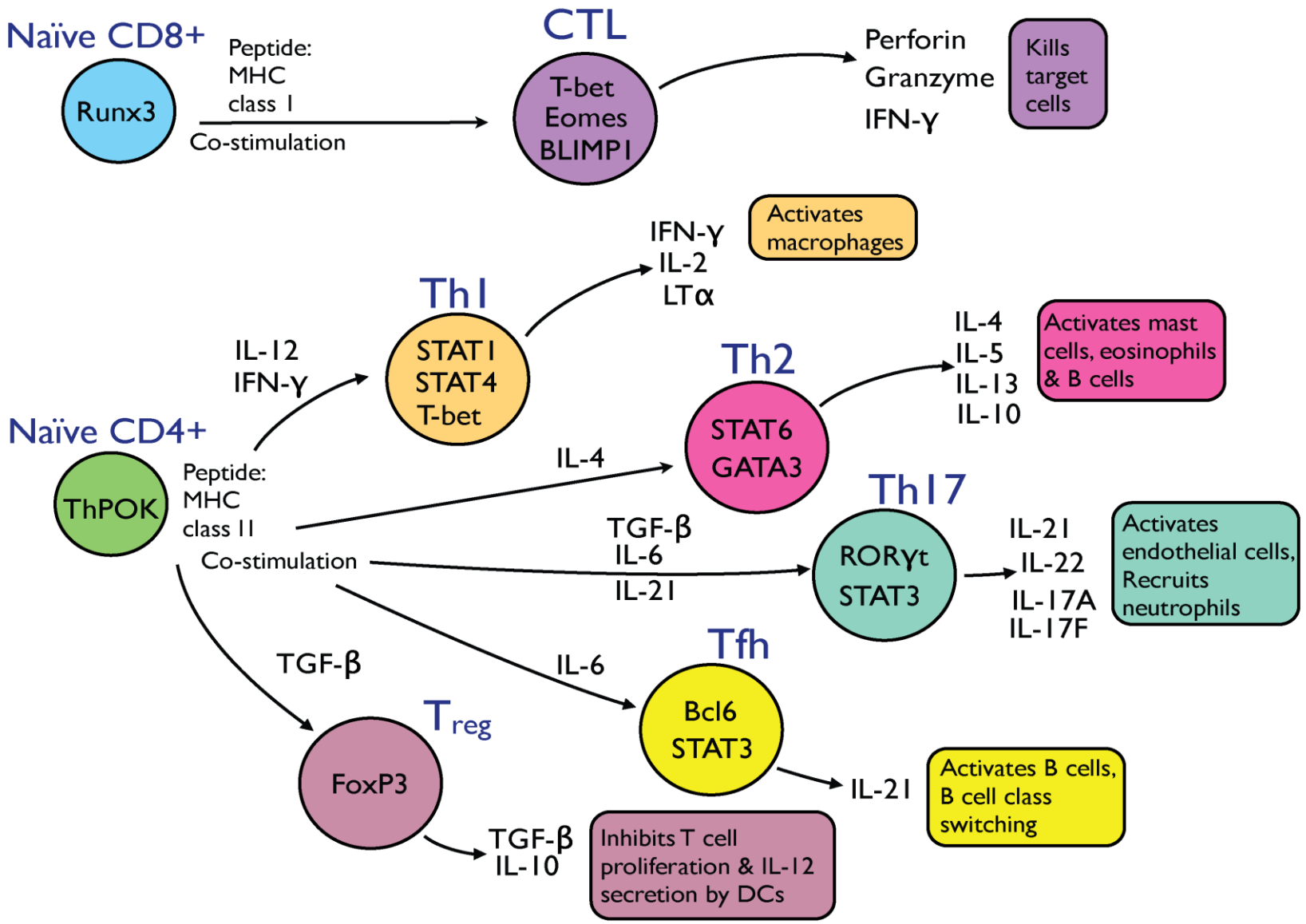
(Acuto and Michel, 2003). T_{reg} cells produce TGF-β and IL-10 (Saraiva and O'Garra, 2010). Both these cytokines can inhibit T cell proliferation, and IL-10 can also inhibit the secretion of IL-12 by DCs (Saraiva and O'Garra, 2010). Recently, subsets of regulatory T cells have been described that lack expression of FoxP3. These include Th3 and Tr1 cells, neither of which have been well characterised (Roncarolo et al., 2001; Saraiva and O'Garra, 2010).

Schematic 1.2 T cell subtypes

This schematic illustrates some of the main T cell effector subtypes that arise upon activation of a naïve $CD4^+$ or $CD8^+$ T cell. The transcription factors responsible for differentiation/maintenance of the T cell subtype are listed inside the cell. The cytokines responsible for promoting differentiation into a particular T cell subtype are shown on an arrow leading to the cell itself. Arrows leading away from the cell show the cytokines that the differentiated cell secretes. Boxes list the main effector functions of the T cell subtype. Naïve T cells normally require interactions with both peptide:MHC and co-stimulatory molecules before they may differentiate into an effector subset. However, if a naïve T cell recognises peptide:MHC in the absence of co-stimulation, then it may become a regulatory T cell (Schwartz, 2003).

DC = dendritic cell. CTL = cytotoxic T lymphocyte.

Schematic created by myself. Inspiration for schematic taken from (Dong and Martinez, 2010).


Naïve CD8+
Runx3
Peptide:
MHC
class I
Co-stimulation
CTL
T-bet
Eomes
BLIMP1
Perforin
Granzyme
IFN-γ
Kills
target
cells
Naïve CD4+
ThPOK
Peptide:
MHC
class II
Co-stimulation
IL-12
IFN-γ
Th1
STAT1
STAT4
T-bet
IFN-γ
IL-2
LTα
Activates
macrophages
IL-4
Th2
STAT6
GATA3
IL-4
IL-5
IL-13
IL-10
Activates mast
cells, eosinophils
& B cells
TGF-β
IL-6
IL-21
Th17
RORγt
STAT3
IL-21
IL-22
IL-17A
IL-17F
Activates
endothelial cells,
Recruits
neutrophils
TGF-β
Treg
FoxP3
TGF-β
IL-10
Inhibits T cell
proliferation & IL-12
secretion by DCs
IL-6
Tfh
Bcl6
STAT3
IL-21
Activates B cells,
B cell class
switching

1.4.3 T cell homeostasis

In the growing mammal, the lymphocyte pool increases in size, only to be maintained at a relatively constant state in the adult by poorly understood homeostatic mechanisms (Takada and Jameson, 2009). T lymphocytes are in constant production, both by the thymus and by peripheral division in response to antigenic stimulation. Quite simply, in order for equilibrium to be maintained, one T cell must die for every new T cell that is made. The ready abundance of T cells in a lymphoreplete individual means that these cells are in constant competition with each other for survival. Essentially, competition for resources acts to shape the T cell pool (Takada and Jameson, 2009).

A T cell's requirement for resources changes throughout its lifespan. This means that different T cell types can occupy separate niches and thus can avoid being in direct competition with one another for space and survival signals (Takada and Jameson, 2009). The thymus, as a primary lymphoid organ and the site of T cell development, has a profound influence on the T cell repertoire. For correct thymocyte differentiation, contact with thymic epithelial cells is necessary and hence, thymic architecture must be maintained. For a thymocyte to be positively selected, it must engage in a low affinity interaction with self-peptide:MHC complexes. Following egress from the thymus, the now mature, but naïve T cells continue to require interactions with self-peptide:MHC in order to survive (Kirberg et al., 1997).

As a naïve T cell changes to a memory state, its requirement for survival signals changes. In contrast to naïve cells, memory T cells are thought not to require TCR:self-peptide:MHC interactions in order to survive (Murali-Krishna et al., 1999; Swain, 2000). Naïve and memory CD8 cells are believed to occupy different competitive niches and therefore may not compete for the same limiting resources (Freitas and Rocha, 2003). However, there is evidence that this is not the case for naïve and CD4 memory cells, particularly in conditions of lymphopenia, when integration of TCR and IL-7 signals seems to favour survival of both cell types (Seddon et al., 2003).

The cytokines that use receptors containing the common γ-chain (γ_c) are particularly important for T cell homeostasis. These consist of IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21. T cell survival requires signalling through JAK3, and this kinase can be activated by all γ_c receptors (Sohn et al., 1998). Of the γ_c cytokines, IL-7 is the most crucial. IL-7 is non-redundant for both the survival and homeostatic proliferation of naïve CD4 and CD8 T cells (Bhatia et al., 1995; Freeden-Jeffry et al., 1995), although the exact mechanism through which IL-7 works is unclear. Under normal circumstances, the levels of IL-7 appear to be limiting - if IL-7 is over-expressed, then the size of the T cell pool can increase (Geiselhart et al., 2001). Interestingly, once activated, a T cell seems to require only one of IL-2, IL-4, IL-7, or IL-15 in order to survive (Vella et al., 1998).

IL-7 is necessary for the survival and homeostatic proliferation of CD4 memory cells (Bradley et al., 2005; Seddon et al., 2003). In comparison, generation of memory CD8 cells is controlled by both IL-7 and IL-15 (Buentke, 2006). The effect that IL-7 has on a T cell is regulated through the expression of the IL-7 receptor (IL-7R). The IL-7R is absent on thymocytes during positive selection and on activated T cells. The strength of TCR signalling during positive selection correlates with the upregulation of the IL-7R on SP thymocytes (Sinclair et al., 2011). New T cells can further upregulate IL-7R expression as they leave the thymus, in a process dependent on activation of the NF-κB transcription factor (Silva et al., 2014).

TCR and IL-7R signals have been found to synergize to induce homeostatic proliferation (Seddon and Zamoyska, 2002). CD4 and CD8 mature T cells die after losing their TCRs (Labrecque et al., 2001; Polic et al., 2001). The TCR clearly induces a T cell survival signal. However, the precise nature of these homeostatic signals is unknown. The Src family kinases Lck and Zap70 are required for homeostatic proliferation (Schim van der Loeff et al., 2014; Seddon et al., 2000). Interestingly, while Fyn expression is not sufficient to induce proliferation, there is redundancy between Lck and Fyn for survival, suggesting that survival and proliferation signals are distinct (Seddon et al., 2000).

1.5 Signalling within the immune system

Cells of the innate and adaptive immune system possess a variety of cell surface receptors. These receptors sense information about the extracellular environment and transduce it to appropriate cell compartments via the activation of intracellular signalling pathways. Signalling pathways can vary enormously, but essentially, they all rely on protein:protein interactions (Pawson and Nash, 2003). In many cases, cell signalling cascades end with the activation of a transcription factor. In such instances, gene expression can occur and proteins may be synthesised. Alternatively, signalling pathways may act upon the cytoskeleton and lead to mobilisation of the cell or changes in shape and size (Murphy, 2011).

Cell surface receptors have an extracellular region to sense the environment and usually, an intracellular region (cytoplasmic tail) to activate the signalling cascade. If the receptor itself lacks the intracellular region, then it must closely associate with a second protein boasting such a region. In order to function, a signalling cascade must employ the activity of enzymes. Protein kinases are an important class of these enzymes (Pawson and Nash, 2003). They add phosphate groups to tyrosine, serine, or threonine amino acid residues. Often protein kinases are associated with the receptor (e.g. the B and T cell receptors) and can be activated rapidly upon ligand binding to the receptor's extracellular portion. Occasionally, the receptor itself has an intrinsic kinase activity within its intracellular region (e.g. the transforming growth factor-β, TGF-β receptor). Such receptors belong to the group of receptor tyrosine kinases or receptor serine/threonine kinases (Pawson and Nash, 2003). Protein kinases tend to activate themselves upon dimerization, before phosphorylating their other protein substrates (Murphy, 2011).

Phosphorylated proteins can easily interact with other proteins. Phosphorylation is also important for activation of many proteins, including enzymes, and for the activation of transcription factors (Murphy, 2011). Dephosphorylation of a protein involves removal of phosphate groups by protein phosphatases and can

be important for terminating or regulating a signalling pathway. Proteins involved in signalling cascades usually contain protein-interaction domains. Such domains, e.g. Src homology 2 (SH2) domains, frequently bind to phosphorylated tyrosines (Pawson and Nash, 2003).

Scaffolds and adaptors are very important components of signalling cascades. They lack any enzymatic activity of their own, but serve to bring other proteins into close proximity with each other (Jordan et al., 2003). Scaffolds are large and can recruit multiple proteins, whilst adaptors are smaller and tend to bring only two other proteins together (Murphy, 2011).

Small GTPases (e.g. Ras, Rac, Rho and Cdc42) can bind to GTP and GDP and are important for signalling downstream of tyrosine kinase associated receptors (Cantrell, 2003). The small GTPases are activated (bind GTP) in response to receptor stimulation. However, they do not stay active for long, and this tight regulation helps to control the magnitude of the signalling response (Cantrell, 2003).

Phosphatidylinositol is an important lipid component of cell membranes (Lemmon, 2003). During receptor signalling it is often phosphorylated, transforming into one of a variety of phosphoinositides (e.g. phosphatidylinositol 3, 4, 5-trisphosphate; PIP_3) (Lemmon, 2003). The phosphoinositides are recognised by the domains of proteins and hence serve to recruit other signalling components to the cell membrane.

Ubiquitination is the process by which ubiquitin chains are added to the lysine residues of protein substrates by ubiquitin ligases. It plays a significant role in many signalling pathways. Polyubiquitination at the K48 residue marks the protein for proteasomal degradation. This aids signal termination (Liu et al., 2005). Conversely, polyubiquitination at the K63 residue can help to propagate the signal by further promotion of protein:protein interactions (Liu et al., 2005).

Within many signalling pathways, enzymatic activity may result in second messenger production. Second messengers enable amplification of the signal

by activation of protein substrates (Lin and Weiss, 2001). Within immune system cells, calcium ions (Ca^{2+}) are important second messengers, which, when released, can lead to activation of the protein calmodulin (Murphy, 2011).

1.5.1 Signalling downstream of the T cell receptor

The T cell receptor (TCR) remains membrane bound, unlike the B cell receptor (BCR), which may be secreted as immunoglobulin. Consequently, for a T cell to fulfil its effector functions, it first requires stimulation of the TCR and activation of downstream signalling cascades.

The TCR is activated when it binds to a peptide:MHC complex on the surface of an APC. Exactly how this first activating signal is transmitted is however, still a matter of some debate. It may involve the clustering of two or more TCRs. It has been proposed that “pseudo-dimeric” MHC:peptide complexes could form on the APC’s surface. These complexes would be composed of one non-self-peptide:MHC molecule and one self-peptide:MHC molecule (Murphy, 2011). In recognising the complex, two separate TCRs may come together and form a dimer. The clustering together of many TCRs (microcluster formation) is also a possibility, although it is unclear whether this could be frequently induced by peptide:MHC complexes (Murphy, 2011; Smith-Garvin et al., 2009). A slightly different possibility is that, upon peptide:MHC binding, the TCR may undergo a conformational change that would help to propagate the signal (Smith-Garvin et al., 2009). During the DN stage of thymocyte development, signalling through the pre-TCR is constitutive and is thought to occur in the absence of ligand (Aifantis et al., 2001).

The TCR α and β-chains (or γ and δ-chains) have only very short cytoplasmic tails. Hence, in order to transmit its signal intracellularly, the TCR must associate closely with the CD3 complex (composed of CD3γ, CD3δ and CD3ε) and with the zeta (ζ) chain (Wucherpfennig et al., 2010) **Schematic 1.3**). Together, the TCR, CD3 complex, and ζ chain form the TCR complex (Wucherpfennig et al., 2010). CD3γ, CD3δ, CD3ε, and the ζ chain all contain

immunoreceptor tyrosine-based activation motifs (ITAMs) in their intracellular regions. The ITAMs each possess two tyrosine residues that, following activation of the TCR, can be phosphorylated by Lck (a member of the Src family of protein tyrosine kinases) (Wucherpfennig et al., 2010). Lck is associated with the CD4 and CD8 co-receptors. For this reason, the binding of CD4 and CD8 to the invariant portions of MHC molecules is a crucial step in TCR signalling (Li et al., 2004). It serves to bring Lck into close proximity to the ITAMs of the TCR complex.

In Lck deficient mice, thymocyte development can progress through the DN stage, but is severely blocked at the DP stage (Molina et al., 1992). Lck signalling is also thought to be important for determining CD4/CD8 lineage commitment during thymocyte development. Stronger or more sustained TCR signalling, mediated by Lck, is believed to lead to CD4 T cell development by enabling expression of the transcription factor ThPOK (He et al., 2005; Singer et al., 2008; Sun et al., 2005). Conversely, it is thought that a weak TCR signal is unable to induce ThPOK, allowing instead for the expression of a second transcription factor, Runx3 and resulting in commitment to the CD8 lineage (Saini et al., 2010; Sinclair et al., 2015).

Once phosphorylated by Lck, the tyrosines residues within the ITAMs of the TCR complex are able bind the SH2 domains of Zap70 (a cytoplasmic protein tyrosine kinase) (Iwashima et al., 1994). Zap70 can then undergo autophosphorylation and can also be phosphorylated by Lck (Chan et al., 1995). In this activated form, Zap70 may phosphorylate LAT (linker of activated T cells, a scaffold protein) and SLP-76 (an adaptor protein) (Huse, 2009). PIP_3, present in the T cell's cytoplasmic membrane, recruits phospholipase C-γ (PLC-γ) by binding to its PH (pleckstrin homology) domain. Once at the membrane, PLC-γ binds to LAT and SLP-76 and may then be activated by Itk (a membrane-associated protein tyrosine kinase) (Samelson, 2003). In naïve T cells, activation of PLC-γ also relies on signals received through the co-stimulatory receptor, CD28 (Murphy, 2011).

Activated PLC-γ causes the breakdown of the membrane lipid phosphatidylinositol bisphosphate (PIP_2) into diacylglycerol (DAG) and 1,4,5-trisphophate (IP_3, a second messenger) (Lin and Weiss, 2001). Ultimately, activated PLC-γ can activate three separate downstream signalling pathways. In one such pathway, IP_3 binds to Ca^{2+} channels in the endoplasmic reticulum (ER). This results in the subsequent release of Ca^{2+} from the ER and into the cytoplasm. The free Ca^{2+} can then bind calmodulin, which in turn binds and activates calcineurin (a serine/threonine phosphatase) (Hogan et al., 2003). Calcineurin dephosphorylates the transcription factor NFAT (nuclear factor of activated T cells), allowing it to enter the nucleus (Hogan et al., 2003).

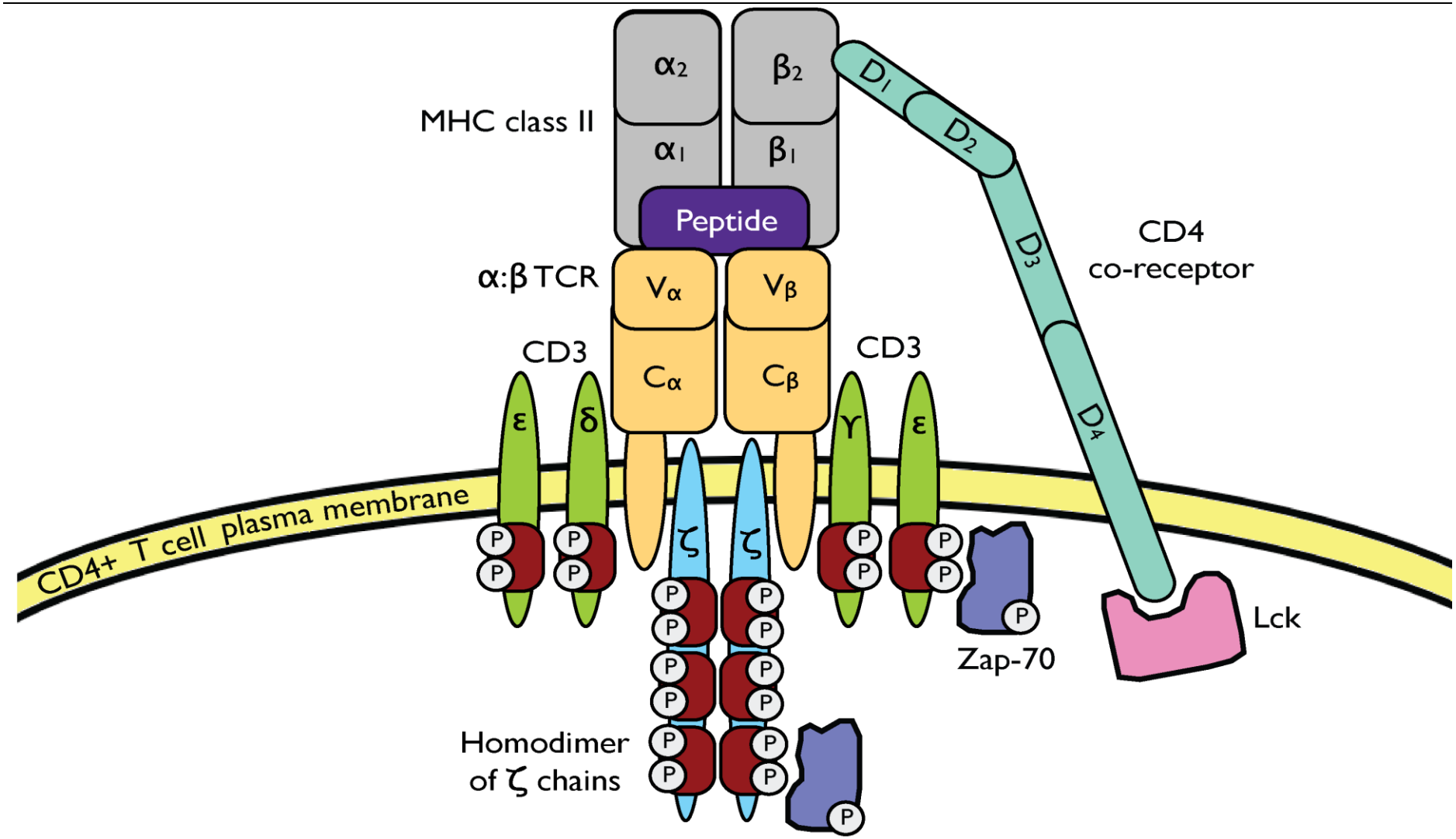
A second pathway initiated by PLC-γ involves the activation of the small GTPase, Ras by DAG (Lin and Weiss, 2001). Ras then activates Raf (a mitogen-activated protein kinase kinase kinase, MAP3K). Raf phosphorylates MEK1 (a MAP2K) and MEK1 phosphorylates Erk (a MAPK). Erk leads to production of the AP-1 transcription factor (Murphy, 2011). The final pathway initiated by PLC-γ involves the recruitment of protein kinase C-theta (PKC-θ) to the membrane by DAG (Kishimoto et al., 1980). PKC-θ can phosphorylate CARMA1 (a scaffold protein) (Rueda and Thome, 2005). This enables recruitment of the CARMA1/Bcl10/MALT1 (CBM) complex to the immunological synapse. The CBM complex can then recruit other proteins, including the IKK (inhibitor of NF-κB kinase) complex and TRAF6 (Rossman et al., 2006; Thome, 2004). Activation of the IKK complex ultimately leads to activation of the transcription factor NF-κB (Rossman et al., 2006). NFAT, AP-1 and NF-κB can all increase the transcription of the cytokine IL-2, which is important for T cell proliferation and differentiation (Murphy, 2011).

Schematic 1.3 The TCR/CD3 complex

The α:β TCR is composed of an α chain and a β chain, each of which has a variable (V) and constant (C) region. The TCR α and β chains have only very short cytoplasmic tails. Therefore, in order to transmit a signal intracellularly, they must associate with the CD3 complex (composed of CD3Ɣ, CD3δ, and CD3ε) and with a homodimer of ζ chains. The TCR, CD3, and ζ chains together form the TCR complex. CD3Ɣ, CD3δ, CD3ε, and the ζ chains all contain immunoreceptor tyrosine-based activation motifs (ITAMs, shown as brown rectangles) in their intracellular regions.

The MHC class II molecule is composed of an α chain and a β chain, each of which has two domains. The amino-terminal domains, α_1 and β_1 are highly polymorphic and form the peptide-binding cleft. The MHC class I molecule (not shown) is composed of an α chain (containing three protein domains) bound to β_2-microglobulin. The CD4 co-receptor is a single chain composed of four Ig-like domains. CD4 binds to the β_2 domain of the MHC class II molecule (Murphy, 2011). The CD8 co-receptor (not shown) is made up of of two separate chains (an α and a β chain), each composed of a single Ig domain. CD8 binds to the α_3 domain of an MHC class I molecule (not shown) (Murphy, 2011). The Src family kinase Lck is associated with the cytoplasmic region of the CD4 (or CD8) co-receptor (Li et al., 2004). When the TCR and co-receptor bind to the MHC molecule, then Lck is brought into close proximity to the ITAMs of the CD3 and ζ chains. Lck phosphorylates the two tyrosine residues present in each of the ITAMs (Wucherpfennig et al., 2010). Another Src family kinase, Zap-70, can then bind to the phosphorylated ITAMs (Iwashima et al., 1994). Zap-70 is phosphorylated by Lck and then phosphorylates other downstream signalling molecules (not shown).

Schematic drawn by myself, but based on and adapted from figures by (Murphy et al., 2011).


MHC class II
α2
β2
α1
β1
Peptide
α:β TCR
Vα
Vβ
Cα
Cβ
CD3
CD3
ε
δ
ζ
ζ
γ
ε
CD4+ T cell plasma membrane
D1
D2
D3
D4
CD4
co-receptor
Lck
Zap-70
Homodimer
of ζ chains
P

1.5.2 Cytokine signalling - the hematopoietin family

The hematopoietin family of cytokines is very large and among others, includes the interleukins 2-7, IL-9, IL-12, and IL-15 (Murphy, 2011). Receptors for the hematopoietin family form dimers upon ligand binding. The cytoplasmic domains of the hematopoietin family receptors are associated with protein tyrosine kinases of the JAK family (Leonard and O'Shea, 1998). Upon cytokine binding and consequent dimerization of the receptor, the JAKs are brought into close proximity with one another (Leonard and O'Shea, 1998). The JAKs first autophosphorylate, before phosphorylating tyrosine residues on the receptor's cytoplasmic tail (Leonard and O'Shea, 1998). The phosphorylated tyrosines of the receptor can interact with the SH2 domains of STATs. At the receptor, the STATs are phosphorylated by the JAKs. The STATs then dimerize, before migrating to the nucleus where they initiate transcription of their target genes (Leonard and O'Shea, 1998).

There are several JAK and STAT proteins, particular combinations of which are associated with signalling from different cytokine receptors. Of note, STAT1 and STAT4 are important for the development of Th1 cells, whilst STAT6 is necessary for Th2 production (Schmitt and Ueno, 2015; Szabo et al., 2003). Cytokines can have dramatic effects on the immune system, and hence their signalling must be tightly regulated. Regulation may be provided by phosphatases (e.g. CD45) or by inhibitors such as the suppressor of cytokine signalling (SOCS) proteins (Yasukawa et al., 2000).

1.5.2.1 IL-7 signalling

IL-7, although initially identified for its role in B cell development, has proven essential to the development and homeostasis of T cells. *Il7* and *Il7rα* knockout mice lack both B and T cells (Freeden-Jeffry et al., 1995; Peschon et al., 1994). Use of neutralising antibodies towards IL-7 has been shown to reduce T cell numbers in the thymus and periphery (Grabstein et al., 1993). Conversely, addition of recombinant IL-7 can increase T cell numbers (Geiselhart et al.,

2001). The IL-7R is composed of the γ_c and the IL-7R α-chain. JAK3 is associated with the γ_c, whilst JAK1 is associated with the IL-7R α-chain (Palmer et al., 2008). This combination of JAKs most frequently leads to activation of STAT5 (Palmer et al., 2008). The STAT5 transcription factor can induce transcription of B cell leukaemia/lymphoma 2 (Bcl-2) (Hand et al., 2010). Bcl-2 is an important anti-apoptotic protein and its expression is believed to be crucial for T cell survival (Borner, 2003).

1.5.3 Apoptosis

Apoptosis is a form of programmed cell death that plays an important role in the immune system. Notably, it is employed in the removal of non-functional or self-reactive T cells and in the disposal of effector T cells following the resolution of an infection (Siegel, 2006). Apoptosis is characterised by plasma membrane blebbing, redistribution of membrane lipids, and degradation of nuclear DNA (Elmore, 2007). Two main pathways can lead to the induction of apoptosis. These are termed the intrinsic and extrinsic pathways (**Schematic 1.4**). Both pathways rely on the activation of aspartic-acid-specific cysteine proteases (caspases) (Siegel, 2006). Caspases are produced in an inactive pro-caspase form and must be activated by cleavage. Initiator caspases cleave the pro-forms of other caspases. Executioner caspases cleave other proteins within the cell, leading to chromosomal degradation (Siegel, 2006).

1.5.3.1 The intrinsic apoptosis pathway

The intrinsic apoptosis pathway is triggered in response to a variety of cellular insults, such as ultra-violet irradiation, heat, hypoxia, and chemotherapeutic drugs, or in response to a lack of survival factors such as IL-7 (Elmore, 2007; Strasser, 2005). Members of the Bcl-2 family regulate the intrinsic apoptosis pathway. Bcl-2 family members possess Bcl-2 homology (BH) domains (Borner, 2003). The family contains both pro-apoptotic and anti-apoptotic members. Certain pro-apoptotic family members (e.g. Bax, Bak and Bok, also known as executioners) can bind to the membrane of mitochondria, causing the

mitochondria to swell. This results in the release of cytochrome c from the mitochondria and into the cytoplasm (Borner, 2003). Once in the cytoplasmic, cytochrome c can bind to Apaf-1 (apoptotic protease activating factor-1). This cytochrome c:Apaf-1 complex provides a site for the recruitment of pro-caspase-9. Pro-caspase-9 is then able to cleave itself into active caspase-9 (Russell and Ley, 2003). Ultimately, caspase-9 enables activation of the effector caspases 3, 6, and 7. Finally, nuclear DNA is cleaved and the cell dies (Russell and Ley, 2003) **Schematic 1.4**).

The anti-apoptotic Bcl-2 family members (e.g. Bcl-2, Bcl-X_L, and Bcl-W, also known as protectors) work to inhibit the pro-apoptotic members (Borner, 2003). The BH3-only members of the Bcl-2 family (e.g. Bim, Bid, Bad, and Bmf, also known as sentinels) are a second group of pro-apoptotic proteins (Strasser, 2005). They do not cause the direct release of cytochrome c, but instead can induce cell death by increasing the activity of the executioners and by inhibiting the protectors (Murphy, 2011).

1.5.3.2 The extrinsic apoptosis pathway

The extrinsic apoptosis pathway is initiated by the binding of an extracellular ligand to a death receptor. The death receptors are death domain (DD) containing members of the TNFRSF (Aggarwal, 2003). Within the immune system, Fas (CD95) and TNFR1 are particularly important death receptors (Russell and Ley, 2003).

1.5.3.2.1 FasL signalling pathway

Fas ligand (FasL) is produced as a trimer and is present in large amounts on the surface of cytotoxic T cells (Aggarwal, 2003). FasL can induce the trimerization of Fas (**Schematic 1.4**). This brings the death domains of Fas into close contact and enables them to recruit FADD (Fas-associated protein with death domain, an adaptor) (Russell and Ley, 2003). Fas and FADD bind to each other via their death domains (Chinnaiyan et al., 1995). FADD also

contains a death effector domain (DED), which can bind to the DED present in pro-caspase-8 and (in humans, but not in mice) pro-caspase-10 (Siegel, 2006). The pro-caspases can activate themselves and can then serve to activate the effector caspases 3, 6, and 7 (Siegel, 2006). Cell death results.

1.5.3.2.2 TNF signalling pathway

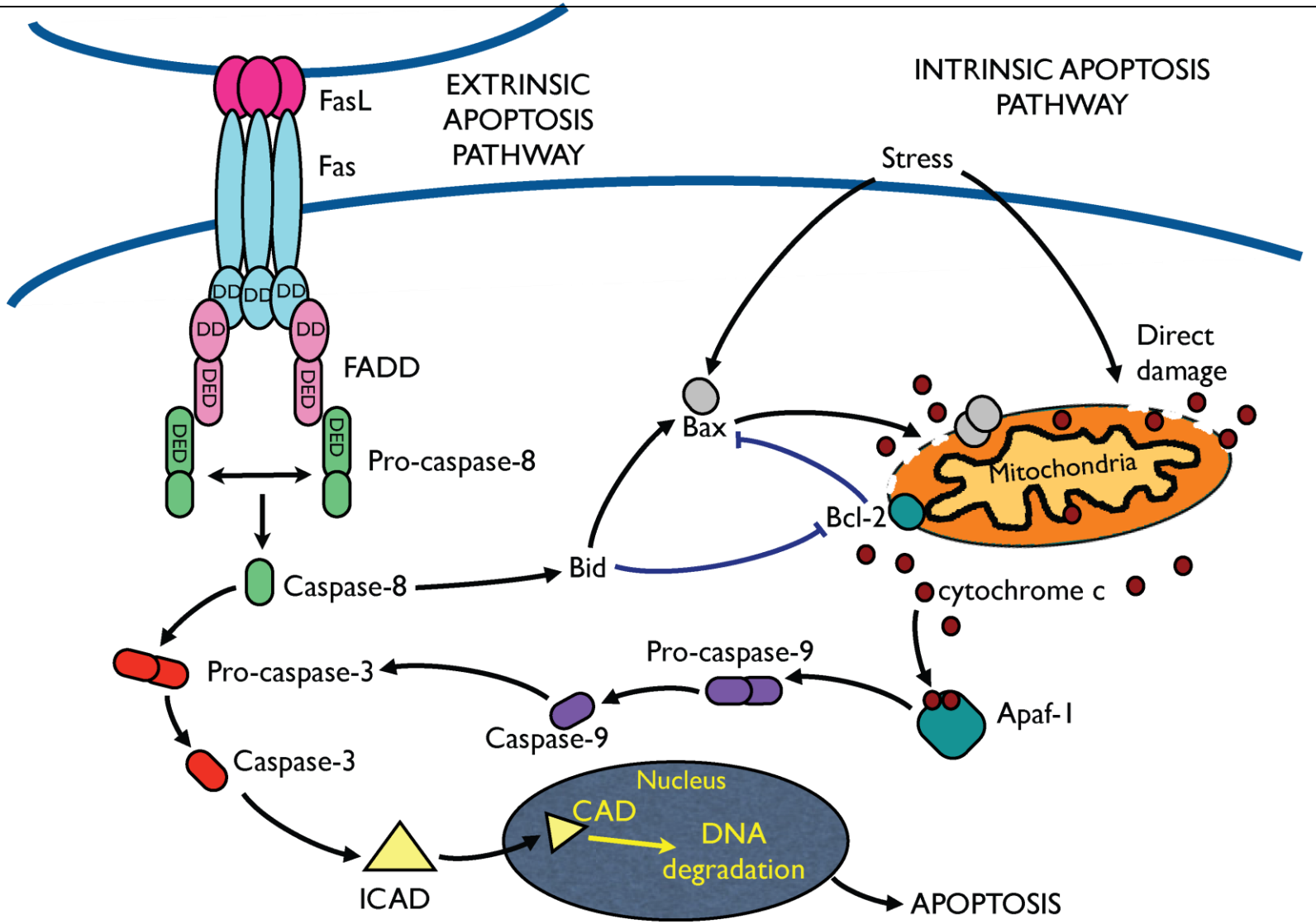
Like Fas, TNF is also produced as a transmembrane trimeric protein. However, unlike Fas, it may additionally be released as a soluble form. Cleavage of TNF from the cell membrane is performed by TNF alpha converting enzyme (TACE) (Black et al., 1997). TNF binding causes timerization of TNFR1. The DDs present on the cytoplasmic tails of TNFR1 can then bind to the DDs present on TRADD (TNFR1 associated death domain protein, an adaptor) (Hsu et al., 1995). Via DD to DD binding, TRADD may recruit FADD (Hsu et al., 1996b). Once FADD is bound, activation of caspases and cell death can proceed, as for the FasL signalling pathway. However, the main function of TNF is not to induce apoptosis, but rather to contribute towards inflammation (Aggarwal, 2003). Indeed, TNF signalling rarely results in cell death. This is because the binding of TRADD to TNFR1 can also initiate a FADD independent signalling pathway (Hsu et al., 1996b). This second pathway terminates in NF-κB activation and results in the transcription of pro-inflammatory and pro-survival proteins. It is believed that the ultimate fate of a TNF stimulated cell depends on the balance between these two pathways (Aggarwal, 2003). In addition to the extrinsic apoptosis pathway and the NF-κB pathway, TNFR1 can also initiate activation of the JUN N-terminal kinase (JNK) and p38 MAPK pathways (Aggarwal, 2003).

Schematic 1.4 Extrinsic and intrinsic apoptosis pathways

The extrinsic apoptosis pathway is activated by the binding of an extracellular ligand to a death receptor. FasL expressed on the surface of cytotoxic T cells can induce trimerization of Fas on the target cell. The death domains (DDs) of Fas are then brought into close contact with one another, enabling them to bind to the DDs of the adaptor molecule FADD (Russell and Ley, 2003). The death effector domain (DED) of FADD can then bind to the DED of pro-caspase-8 (Siegel, 2006). The bound pro-caspase-8 activates itself by proteolytic cleavage (Chang et al., 2003). In turn, the active caspase-8 recruits and activates caspase-3, an effector caspase (Siegel, 2006) and can also lead to the truncation of Bid (Bruin et al., 2008). Caspase-3 cleaves ICAD (inhibitor of caspase activated DNase), mediating the release of CAD and resulting in the degradation of genomic DNA and subsequent death of the cell by apoptosis (Enari et al., 1998).

The intrinsic apoptosis pathway is triggered in response to environmental stress. Stress can either cause direct damage to the mitochondrial membrane or can lead to the activation of Bax (Bruin et al., 2008). The extrinsic apoptosis pathway can also help to activate Bax. Bax can oligomerize at the outer mitochondrial membrane and cause it to swell and leak its contents. Bcl-2 inhibits Bax, while Bid inhibits Bcl-2 (Murphy, 2011). Cytochrome c leaks out from the damaged mitochondria and binds to Apaf-1 present in the cell's cytoplasm. The cytochrome c: Apaf-1 complex can recruit pro-caspase-9. Pro-caspase-9 cleaves itself into active caspase-9, which then leads to the activation of caspase-3 (Russell and Ley, 2003). CAD is released and apoptosis ensues as for the extrinsic apoptosis pathway.

Schematic drawn by myself.


EXTRINSIC
APOPTOSIS
PATHWAY
INTRINSIC APOPTOSIS
PATHWAY
FasL
Fas
DD
DED
FADD
Pro-caspase-8
Caspase-8
Bid
Stress
Direct
damage
Bax
Bcl-2
Mitochondria
cytochrome c
Apaf-1
Pro-caspase-9
Caspase-9
Pro-caspase-3
Caspase-3
ICAD
Nucleus
CAD
DNA
degradation
APOPTOSIS

1.6 The NF-κB pathway

1.6.1 An introduction to the NF-κB pathway

Nuclear factor kappa B (NF-κB) was discovered in the laboratory of David Baltimore in 1986. It was described as a factor present in the nucleus of B cells, bound to the enhancer of the kappa light chain of immunoglobulin (Sen and Baltimore, 1986). Later experiments revealed that NF-κB signalling takes place in virtually all mammalian cells, being either constitutively active or rapidly induced when required. This signalling pathway is very complicated – it is activated by numerous stimuli, regulated by many mechanisms, and leads to the transcription of many genes. So finely balanced is this cascade that any improper regulation will, and frequently does, lead to pathology (Pasparakis et al., 2006).

1.6.1.1 The NF-κB/Rel and IκB families

The NF-κB/Rel family of transcription factors consists of RelA (p65), c-Rel, RelB, NF-κB1 (p50/p105), and NF-κB2 (p52/p100). These proteins all display a 300 amino acid long Rel homology domain (RHD) at their N-terminus. Via this domain, the proteins form homo or heterodimers with one another, thus creating the NF-κB transcription factor (Gerondakis et al., 2013). The dimers are held in the cytoplasm by inhibitory proteins, the inhibitors of NF-κB (IκBs). These include IκBα, IκBβ, IκBε, and the precursor proteins of p50 and p52 - p105 and p100, respectively (Gerondakis et al., 2013). Only when the inhibitory proteins are phosphorylated and subsequently ubiquitinated and degraded, are the NF-κB dimers free to translocate to the nucleus, where they meet their target genes (Gerondakis et al., 2013).

1.6.1.2 The IKK complex

Around 10 years after the discovery of NF-κB, groups started to describe the existence of a large, multisubunit kinase that could phosphorylate the serine

resides of IκBα (Chen et al., 1996; Lee et al., 1997). The kinase activity of the large complex was later found to be stimulus induced, and the complex was renamed IKK (for IκB kinase) (DiDonato et al., 1997). Today, we know that the IKK complex contains two catalytic subunits - IKK1 (IKKα) and IKK2 (IKKβ) - and a regulatory subunit - NF-κB essential modulator (NEMO/ IKKγ/ IKKAP) (Ghosh and Karin, 2002).

The amino acid sequences of IKK1 and IKK2 are 52% identical to each other and even more alike (65%) in their catalytic domains (Ghosh and Karin, 2002). Both kinases act upon the two serine residues present in the N-terminal regulatory domain of the IκB proteins, with IKK2 being more catalytically active than IKK1 (Ghosh and Karin, 2002). IKK1 and IKK2 dimerise via their leucine zipper motifs, usually heterodimers are formed, although homodimers are possible. IKK1/2 heterodimers have been shown to have greater catalytic efficiency than either the IKK1 or the IKK2 homodimer (Huynh et al., 2000). The catalytic dimer contains two short motifs, located at each carboxy terminus, which allow for interaction with NEMO molecules (Huxford and Ghosh, 2009). NEMO is able to link the IKK complex to upstream signalling components via the zinc finger motif in its carboxy terminus (Ghosh and Karin, 2002). The large size (700-900kDa) of the IKK complex indicates the presence of other proteins, for example Cdc37 and Hsp90, the functions of which have not been clearly established (Hayden and Ghosh, 2004). Before the IKK complex can become functional, it must first be activated by phosphorylation of two serine residues located in the “activation loops” of the catalytic subunits (Delhase et al., 1999; Ling et al., 1998).

1.6.1.3 The canonical vs noncanonical pathway of NF-κB activation

When individual IKK subunits were knocked out in cells it became apparent that two different pathways of NF-κB activation exist, the canonical/classical pathway and the noncanonical/alternative pathway (Pasparakis et al., 2006) **Schematic 1.5**). The canonical pathway is activated in response to pro-

inflammatory stimuli, e.g. IL-1, TNF, and LPS. It relies heavily on IKK2 and NEMO and principally regulates p50/RelA and p50/c-Rel dimers. The alternative pathway is quite different and is stimulated in response to specialist receptors (e.g. the BAFF receptor and lymphotoxin-β receptor). It is involved in B cell maturation and lymphoid organogenesis (Pasparakis et al., 2006). IKK1 is critical to this pathway, which involves the processing of the p100 subunit and formation of p52/RelB dimers (Senftleben et al., 2001a). Even in the absence of IKK2, cells are still able to respond weakly to pro-inflammatory cytokines, whilst IKK1/2 double deficient cells lack any signalling (Li et al., 2000). Hence, IKK1 can compensate for the loss of IKK2 to a certain extent. The reverse cannot be said of the alternative pathway, where IKK1 is essential for stimulation induced NF-κB signalling. Thus, it was concluded that the canonical pathway involves an IKK1/IKK2/NEMO complex, whist the alternative pathway relies on IKK1 homodimers (Pasparakis et al., 2006).

Schematic 1.5 The canonical and non-canonical pathways of NF-κB activation

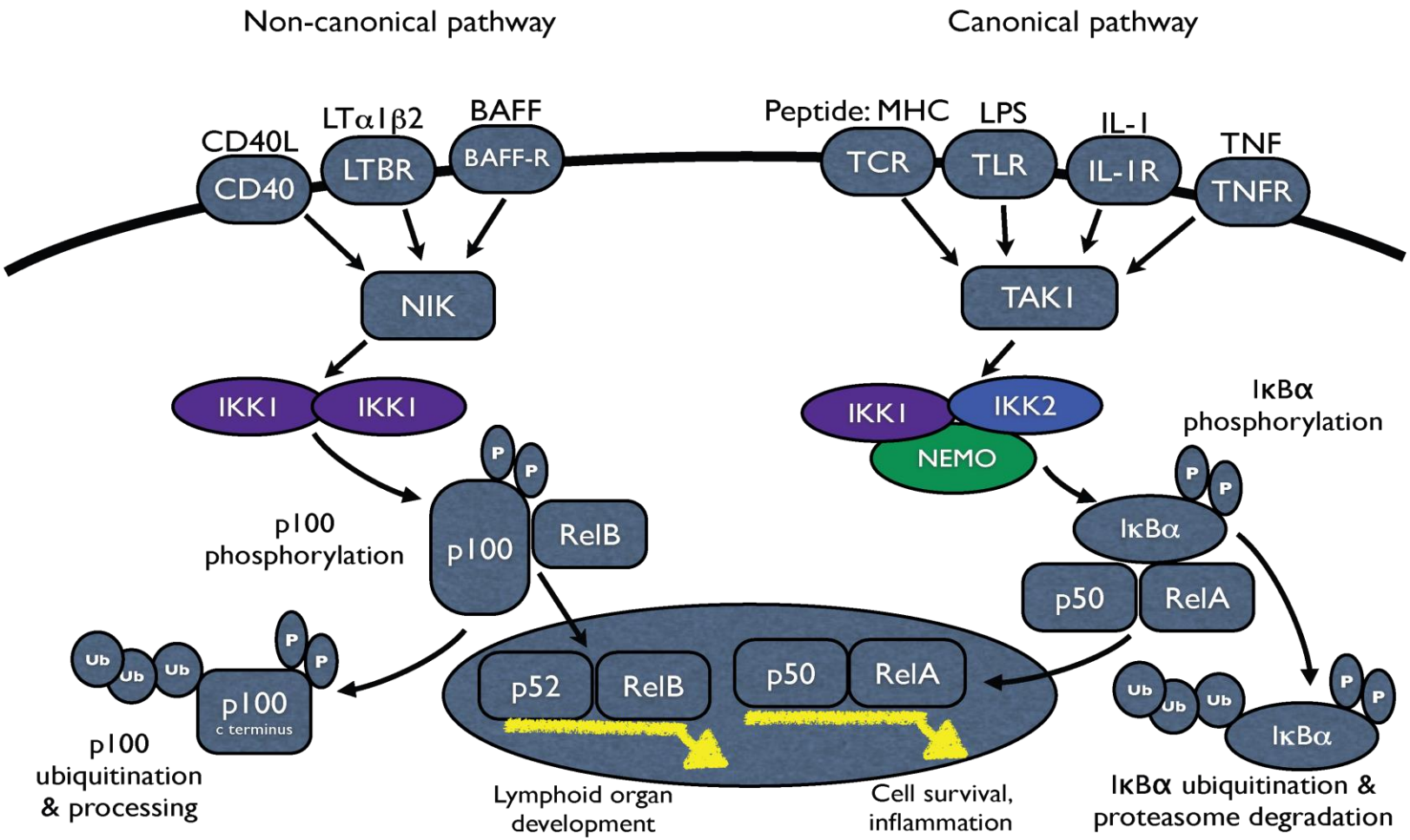
There are two different pathways of NF-κB activation; the canonical (classical) and non-canonical (alternative) pathways. In T cells, the canonical pathway may be activated by peptide:MHC binding to the TCR (Hayden et al., 2006). In a large variety of cells types, the canonical pathway is frequently activated by inflammatory stimuli, including LPS, IL-1, and TNF (Pasparakis et al., 2006). The IKK complex, consisting of IKK1, IKK2, and NEMO, is central to the canonical pathway of NF-κB activation. In the canonical pathway the IKK kinase TAK1 leads to phosphorylation of IKK1 and IKK2 (Sato et al., 2005; Shim et al., 2005). The activated IKK complex is then able to phosphorylate the IκBs (IκBα is shown), allowing for their K48-linked ubiquitination and degradation by the proteasome (Ghosh and Karin, 2002). This frees the NF-κB dimers, allowing them to translocate to the nucleus. The formation of p50/RelA dimers (shown) and p50/c-Rel dimers is common to the canonical pathway (Pasparakis et al., 2006). Activation of the canonical pathway leads to transcription of cytokines, chemokines, and adhesion molecules, thus helping to create a pro-inflammatory environment (Lawrence, 2009). In addition, NF-κB activation through this pathway stimulates production of many anti-apoptotic proteins (Aggarwal, 2003).

The non-canonical pathway tends to be activated downstream of a more limited number of receptors. In B cells, stimulation of the BAFF receptor (BAFF-R) leads to activation of the non-canonical NF-κB pathway (Pasparakis et al., 2006). The lymphotoxin beta receptor (LTBR) is expressed on a variety of cell types (with the exception of T and B cells) and is activated in response to stimulation with the membrane bound form of lymphotoxin (LTα1β2). Activation of the non-canonical pathway via the LTBR plays a vital role during the formation of the peripheral lymphoid organs (Pasparakis et al., 2006). As shown, binding of ligand to the co-stimulatory receptor CD40 also activates the non-canonical NF-κB pathway. An IKK1 homodimer is central to the non-canonical pathway. This is activated by the IKK kinase NIK (Senftleben et al.,

2001a; Xiao et al., 2001). The activated IKK1 homodimer is then able to phosphorylate p100 (the inactive precursor product of the *nfkb2* gene), allowing it to be processed into the functional p52 subunit. In the non-canonical pathway p52 often forms a dimer with RelB (shown) and translocates to the nucleus (Senftleben et al., 2001a).

Schematic drawn by myself.

Canonical & non-canonical NF-κB activation pathways



1.6.2 Activation of the NF-κB pathway

Although a huge variety of signalling pathways lead to activation of NF-κB, virtually all these pathways end with the activation of the IKK complex, the degradation of IκB proteins, and the binding of NF-κB to its target genes (Oeckinghaus et al., 2011). Signalling upstream of the IKK complex varies according to the receptor activated. Nevertheless, several of the signalling components are common to many NF-κB signalling pathways. For example, proteins belonging to the receptor interacting protein kinase (RIPK1, RIP) and TNF receptor associated factor (TRAF) families are often employed in pathways leading to NF-κB activation (Oeckinghaus et al., 2011).

TRAF proteins are usually involved in both canonical and noncanonical NF-κB activation, whilst RIPK proteins seem mostly restricted to the canonical pathway (Hayden and Ghosh, 2008). The noncanonical NF-κB pathway is activated by specific members of the TNFRSF, including the lymphotoxin-β receptor, the BAFF-R, and CD40. In contrast, the canonical NF-κB pathway is activated downstream of numerous receptors, including the B and T cell receptors, the Toll/IL-1 receptors, and the TNF receptor (Hayden et al., 2006).

1.6.2.1 Common signalling components upstream of IKK

1.6.2.1.1 The TRAF family

TRAF protein family members, of which there are 7, are defined by their C-terminal TRAF domain. The TRAFs are adaptors, but TRAF family members 2-7 have a RING domain and are thought capable of functioning as E3 ubiquitin ligases (Oeckinghaus et al., 2011). While both TRAF2 and TRAF5 are believed to be important in the TNF signalling pathway, TRAF6 has been implicated in TCR and Toll/IL-1 signalling (Oeckinghaus et al., 2011). In the noncanonical NF-κB pathway, TRAF3 may act as a negative regulator of signalling, requiring degradation by other TRAF family members (Hayden and Ghosh, 2008). TRAF proteins may recruit the IKK complex by binding directly to IKK1 and IKK2

(Devin et al., 2001), however, evidence suggests that other proteins are usually also involved in IKK recruitment (Hayden and Ghosh, 2008). For example, during TNF signalling, RIPK1 is believed to be important in helping to recruit IKK and, during TCR signalling, PKC family members are known to play a role in IKK recruitment (Hayden and Ghosh, 2008).

1.6.2.1.2 The RIPK family

In an NF-κB signalling pathway, RIPKs usually work together with TRAFs, or upstream of TRAFs, to activate IKK (Hayden and Ghosh, 2008). There are 7 members of the RIPK family, all of which possess a serine/threonine kinase domain. RIPK1 also contains a death domain (DD), which can interact with the death domains of adaptors and receptors (Oeckinghaus et al., 2011). RIPK2 contains a CARD (caspase activation and recruitment) domain and is utilised in signalling pathways activated by the NODD-LRR family of intracellular pattern recognition receptors (Hayden and Ghosh, 2008). RIPK3 is thought to be involved in many NF-κB signalling pathways, and there is evidence that it may inhibit RIPK1 induced NF-κB activation (Hayden and Ghosh, 2008). Both RIPK1 and RIPK2 can recruit the IKK complex through NEMO - RIPK1 binds via its DD and RIPK2 via its CARD domain (Hayden and Ghosh, 2008). The kinase activity of RIPK1 and RIPK2 seems dispensable for many NF-κB activating pathways (Hasegawa et al., 2008; Lee et al., 2004). Instead they seem to function mostly as scaffolds to enable IKK activation.

1.6.2.1.3 IKK kinases (IKK-Ks)

IKK kinases (IKK-Ks) are required downstream of RIPKs and TRAFs and are necessary for the phosphorylation and subsequent activation of IKK (Hayden and Ghosh, 2008). TAK1 (TGF-β activated kinase 1) is the most common IKK-K of the canonical NF-κB pathway (Sato et al., 2005; Shim et al., 2005). Notably, TAK1 deficient mice lack TNF induced IKK activation (Liu et al., 2006). In the noncanonical pathway, NIK (NF-κB-inducing kinase) seems to be the IKK-K required for IKK1 activation (Senftleben et al., 2001a; Xiao et al., 2001).

1.6.2.2 Important signalling pathways that lead to NF-κB activation

1.6.2.2.1 TNF signalling to NF-κB

The TNFSF currently includes 19 cytokines, which bind to 29 receptors (Aggarwal et al., 2012). Signalling induced by members of the TNFSF can have dramatic effects on the immune system. TNF is the most well characterised family member and is a well-known inducer of the canonical NF-κB pathway. In most cell types, TNF causes cell activation, inflammatory cytokine production and transcription of anti-apoptotic proteins (Aggarwal et al., 2012). However, if NF-κB signalling is blocked, then exposure to TNF frequently causes death by the extrinsic apoptosis pathway (Aggarwal et al., 2012). Dysregulated TNF signalling has been implicated in a number of diseases. For this reason, anti-TNF antibodies have been developed for clinical use. Today such antibodies are proving particularly useful in the treatment of rheumatoid arthritis (Aggarwal, 2003). However, it is hoped that a better understanding of TNF induced NF-κB signalling will lead to improved drug design.

Binding of TNF to TNFR1 induces receptor trimerisation (**Schematic 1.6**). The silencer of death domain (SODD) can then dissociate from the receptor, allowing TRADD to bind (Jiang et al., 1999). TRADD recruits TRAFs to the receptor complex, with TRAF2 believed to be the most important (Hsu et al., 1996b). Evidence suggests that TRAF2 recruits the IKK complex to the receptor by binding directly to both IKK1 and IKK2 (Devin et al., 2001). RIPK1 is also recruited via TRADD (Hsu et al., 1996a). RIPK1 can then bind to the IKK complex through NEMO (Zhang et al., 2000). It is believed that, by acting as a scaffold, RIPK1 also enables recruitment of TAK1, thus bringing an IKK-K into close proximity to IKK (Lee et al., 2004). Evidence suggests that the K63-linked ubiquitination of RIPK1 is necessary for recruitment of the IKK complex and TAK1 (Ea et al., 2006). It was initially thought that such ubiquitination may be provided by TRAF2 (Hayden and Ghosh, 2012), however, more recent evidence has implicated cellular inhibitor of apoptosis 1 (cIAP1) and 2 (cIAP2)

(Yin et al., 2009). Whilst bound to RIPK1, TAK1 phosphorylates and activates IKK. IκB proteins are then degraded, and NF-κB dimers are freed. TNF induced degradation of IκB is very rapid and is believed to take around 10 minutes (Hayden and Ghosh, 2004).

1.6.2.2.2 TCR signalling to NF-κB

Engagement of the TCR and its co-receptors results in the immediate recruitment of Lck and Zap70 and later to the production of DAG. DAG can then recruit PKC-θ to the site of the immunological synapse. PKC-θ phosphorylates CARMA1 causing activation and oligomerization of the CBM complex, which consists of CARMA1, Bcl-10 and MALT1 (McAllister-Lucas et al., 2001). CARMA1 and Bcl-10 are both CARD containing proteins, and MALT contains a DD. Mice deficient in CARMA1 show poor antigen receptor induced activation of NF-κB (Egawa et al., 2003; Wang et al., 2002).

It remains poorly understood precisely how the CBM complex is linked to activation of IKK. However, it is believed that clusters of Bcl-10 and MALT1 may dissociate from the CBM complex (**Schematic 1.6**). These clusters are termed POLKADOTS and are enriched for the presence of TRAF6 (Rossman et al., 2006). Activated TRAF6 causes the K63-linked ubiquitination of NEMO (Deng et al., 2000). Ubiquitinated NEMO can then recruit TAK1, leading to the phosphorylation of IKK2 (Sun et al., 2004). The activated IKK complex proceeds as normal, degrading IκB proteins and allowing NF-κB to translocate to the nucleus. TCR signalling induced degradation of IκB has been shown to require approximately 45 minutes (Hayden and Ghosh, 2004). Of note, co-stimulatory signals mediated by CD28 can also lead to PKC-θ recruitment and hence can increase NF-κB activity within the T cell following APC recognition (Gerondakis et al., 2013).

1.6.2.2.3 Toll/IL-1 signalling to NF-κB

Toll-like receptor (TLR) induced activation of NF-κB is especially important in the early stages of an immune response. Phagocytic cells of the innate immune system and epithelial cells are well known carriers of TLRs. However, T cells can also express some TLRs. In particular, TLR2, TLR3, TLR5, and TRL9 are believed to act as co-stimulatory molecules, which can aid T cell proliferation or cytokine production (Kabelitz, 2007). 13 mammalian TLRs have been identified, all of which can activate NF-κB (Takeda and Akira, 2015). Since the intracellular domain of TLRs is highly homologous to that of the IL-1R, it is referred to as the Toll-IL1R (TIR) domain.

In drosophilia, Toll is expressed by hemocytes and fat body cells, both of which are phagocytic and form part of the insect's immune system (Minakhina and Steward, 2006). The signalling pathway activated by ligation of Toll shares similarities with the Toll/IL-1 signalling pathway of the vertebrate immune system and results in the activation of a transcription factor homologous to NF-κB. Drosophilia have 3 NF-κB homologs: Dorsal, Dif, and Relish, all of which contain the Rel homology domain (Minakhina and Steward, 2006). The highly conserved nature of NF-κB signalling pathways within the immune system is surely indicative of their importance.

Ligand binding causes dimerization of the Toll-IL-1R (**Schematic 1.6**). This brings the intracellular TIR domains into close contact and enables them to interact with the TIR domains of adaptor proteins (Murphy, 2011). The adaptors downstream of the Toll-IL-1R include MyD88, MAL, TRIF, and TRAM (Honda and Taniguchi, 2006). There are two major pathways induced by Toll-IL-1Rs, the MyD88-dependent and the TRIF-dependent pathway (Takeda and Akira, 2015). All receptors, with the exception of TLR3, can activate NF-κB via the MyD88 pathway (Takeda and Akira, 2015). The DD of MyD88 recruits the serine/threonine kinases IRAK1 and IRAK4 (Hiscott et al., 2006). The activated IRAKs can then recruit the E3 ubiquitin ligase TRAF6. TRAF6 acts together with the E2 ligase TRICA1, causing K63-liked ubiquitination of TRAF6 and NEMO

(Murphy, 2011). TAK1 can bind to the ubiquitin scaffold provided by TRAF6 and can be phosphorylated by IRAK (Qian et al., 2001). Activated TAK1 can then phosphorylate IKK2 (Sun et al., 2004). The pathway proceeds with degradation of the IκBs and NF-κB induced transcription.

Schematic 1.6 IL-1R, TCR, and TNFR1 induced activation of the NF-κB signalling pathway

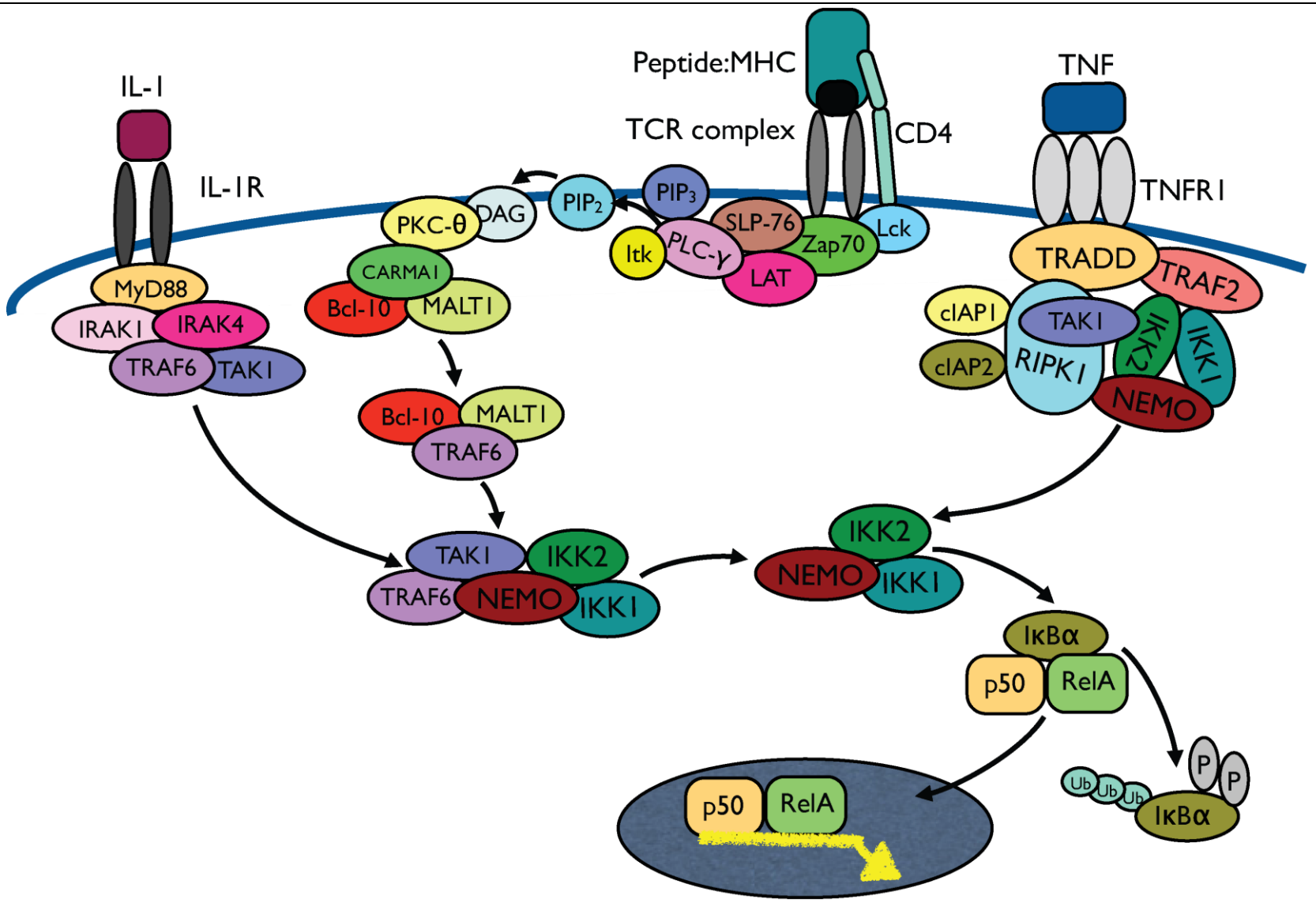
IL-1 cytokine binding to the IL-1R can activate the NF-κB signalling pathway. IL-1 binding causes dimerization of the IL-1R, thus bringing the TIR domains of the receptor into close contact with one another and allowing them to interact with the TIR domains present on the adaptor MyD88 (Murphy, 2011). The death domain of MyD88 can then recruit the serine/threonine kinases IRAK1 and IRAK4 (Hiscott et al., 2006). The activated IRAKs can recruit the E3 ubiquitin ligase TRAF6 (Murphy, 2011). The IKK kinase TAK1 can bind to TRAF6 and can be activated by the IRAKs (Qian et al., 2001). TRAF6 can cause the K63-linked ubiquitination of NEMO (Deng et al., 2000). Ubiquitinated NEMO can also recruit TAK1 (Sun et al., 2004). The activated TAK1 can then phosphorylate and activate the IKK complex. The activated IKK complex phosphorylates the IκBs, leading to the release of NF-κB dimers, which then translocate to the nucleus. The intracellular domain of the IL-1R is highly homologous to that of the toll-like receptors (TLRs). Thus TLR stimulation can also activate NF-κB signalling in this manner.

Peptide:MHC binding to the TCR complex may activate the NF-κB signalling pathway. Activation of the TCR complex results in the immediate recruitment of the protein tyrosine kinase Lck (closely associated with the CD4 or CD8 co-receptor - here shown as CD4) to the ITAMs of the TCR complex. The protein tyrosine kinase Zap70 is then recruited to the ITAMs and is activated by autophosphorylation and phosphorylation by Lck (Chan et al., 1995). Activated Zap70 phosphorylates LAT (a scaffold) and SLP-76 (an adaptor) (Huse, 2009). PIP_3, in the T cell's cytoplasmic membrane, can recruit PLC-γ, which then binds to LAT and SLP-76 at the receptor complex. The protein tyrosine kinase Itk can then activate PLC-γ (Samelson, 2003). Activated PLC-γ breaks down the membrane lipid PIP_2 into DAG and the second messenger IP_3 (not shown). DAG recruits PKC-θ to the membrane (Kishimoto et al., 1980). PKC-θ then phosphorylates the scaffold protein CARMA1, enabling recruitment of the CBM (CARMA1/Bcl-10/MALT1) complex to the immunological synapse (McAllister-

Lucas et al., 2001). Clusters of Bcl-10 and MALT1 (termed POLKADOTS) are believed to dissociate from the CBM complex and to be enriched for the presence of TRAF6 (Rossman et al., 2006). TRAF6 causes the K63-linked ubiquitination of NEMO (Deng et al., 2000). Ubiquitinated NEMO can then recruit TAK1 (Sun et al., 2004). TAK1 activates the IKK complex, leading to the degradation of the IκBs and the translocation of the NF-κB transcription factor.

TNF cytokine binding to TNFR1 can activate the NF-κB signalling pathway. When TNF cytokine binds to TNFR1, conformation changes are induced on the receptor. This enables recruitment of TRADD to the death domain of TNFR1 (Hsu et al., 1995). TRADD then recruits RIPK1 and TRAF2 (Hsu et al., 1996a; 1996b). TRAF2 interacts with IKK1 and IKK2 of the IKK complex (Devin et al., 2001), whilst RIPK1 interacts with NEMO (Zhang et al., 2000). RIPK1 also interacts with TAK1, bringing TAK1 into close proximity to the IKK complex (Ea et al., 2006). cIAP1 and cIAP2 are believed to enable K63-linked ubiquitination of RIPK1 (Yin et al., 2009), which is necessary for the recruitment of both the IKK complex and TAK1 (Ea et al., 2006). The multi-protein structure formed around TNFR1 is called complex I. Complex I mediated activation of the IKK complex results in phosphorylation of the IκBs, allowing for their K48-linked ubiquitination and degradation by the proteasome. This frees the NF-κB dimers, allowing them to translocate to the nucleus.

Schematic drawn by myself.


IL-1
IL-1R
MyD88
IRAK1
IRAK4
TRAF6
TAK1
Peptide:MHC
TCR complex
CD4
PIP2
PIP3
DAG
PKC-θ
CARMA1
Bcl-10
MALT1
Itk
PLC-γ
SLP-76
Zap70
Lck
LAT
Bcl-10
MALT1
TRAF6
TAK1
IKK2
TRAF6
NEMO
IKK1
TNF
TNFR1
TRADD
TRAF2
cIAP1
cIAP2
TAK1
RIPK1
IKK2
IKK1
NEMO
IKK2
NEMO
IKK1
IκBα
p50
RelA
p50
RelA
Ub
Ub
Ub
P
P
IκBα

1.6.3 Manipulating the NF-κB pathway in mice

Over the years, mouse models have proved particularly useful for studying the NF-κB pathway. Gene knockouts (both conventional and conditional), the overexpression of pathway activators and inhibitors, gene knock-ins, and NF-κB reporter genes have all contributed to our understanding of the signalling cascade (Pasparakis et al., 2006).

1.6.3.1 The embryonic/perinatal lethality of IKK and RelA global knockouts

Since the numerous NF-κB signalling pathways all converge upon the IKK complex, it was realised that IKK knockout mice could prove useful in studying the effects of NF-κB blockade. Rel family member knockout mice have also been extensively studied. However, the large amount of redundancy between Rel family members means that single knockouts always have an incomplete block in NF-κB signalling.

Although *Ikk1*$^{-/-}$ mice survive gestation, they die shortly after birth due to numerous morphological defects. They show poor differentiation of the epidermal layer of the skin and skeletal abnormalities, most notably a failure to extend their limb buds (Hu et al., 1999; Li et al., 1999a; Takeda et al., 1999). Fibroblasts from the *Ikk1*$^{-/-}$ mice have been reported as having normal NF-κB activation in response to IL-1 and TNF (Hu et al., 1999; Takeda et al., 1999), although one study did find NF-κB activity to be diminished (Li et al., 1999a). Importantly, the *Ikk1*$^{-/-}$ mice can be rescued by knocking in a mutant IKK1, in which the serines of the activation loop have been have been replaced by alanines (Cao et al., 2001; Hu et al., 2001). This indicates that the kinase activity of IKK1 is not required for its role in skeletal and epidermal development, and hence the morphological defects observed in *Ikk1*$^{-/-}$ mice are independent of NF-κB.

Ikk2$^{-/-}$ mice die during mid-gestation (around E13-14.5) due to extensive TNF induced liver apoptosis (Li et al., 1999c; Tanaka et al., 1999). The lethal phenotype of the *Ikk2*$^{-/-}$ mice can be rescued by blocking TNF signalling via deletion of TNFR1 (Li et al., 1999b). Embryonic fibroblasts from *Ikk2*$^{-/-}$ mice show hardly any NF-κB binding activity in response to TNF or IL-1 (Li et al., 1999c). Such experiments revealed that IKK2 is important for inducing canonical NF-κB signalling in response to TNF and for protecting hepatocytes from TNF induced death. NEMO deficient mice die around E12.5-13, again due to hepatocyte apoptosis. Embryonic fibroblasts from *Nemo*$^{-/-}$ mice show no NF-κB activation in response to TNF, IL-1, or LPS (Rudolph et al., 2000). Like NEMO knockout mice, IKK1/2 double knockout mice also show a complete lack of NF-κB activation and ultimately die at E12 due to liver apoptosis (Li et al., 2000). However, in addition, they show a defect in neurulation, caused by excessive apoptosis of neurons (Li et al., 2000).

RelA$^{-/-}$ mice die at E15-16 due to liver apoptosis (Beg et al., 1995). They can be rescued by breeding onto either a TNF deficient (Doi et al., 1999) or TNFR1 deficient background (Alcamo et al., 2001). However, both RelA/TNF double deficient and RelA/TNFR1 double deficient mice die a few weeks after birth due to infection (Alcamo et al., 2001; Doi et al., 1999).

1.6.3.2 B cell defects in Rel knockout mice

The lethal phenotypes of the IKK knockout and RelA knockout mice had made it difficult for investigators to study the effects of NF-κB deficiency within the immune system. However, mice deficient in NF-κB1 (lacking p105 and p50), NF-κB2 (lacking p100 and p52), RelB, or c-Rel were all found to be viable. Since NF-κB was initially discovered in B cells, early studies of Rel family member deficient mice tended to focus on B cell defects.

B cells from NF-κB1 deficient mice were found to have defects in antibody production and failed to respond to LPS (Sha et al., 1995). Mice deficient in NF-κB2 had defects in the architecture of the spleen and Peyer's patches and

improper formation of B cell follicles and germinal centres (Caamano et al., 1998; Franzoso et al., 1998; Paxian et al., 2002). LTβ induced NF-κB signalling, via the noncanonical pathway, is important for secondary lymphoid organ development. The phenotype of the NF-κB2 deficient mice served to highlight the critical role of the p52 subunit in noncanonical signalling. Like the *Nfκb2*$^{-/-}$ mice, *Relb*$^{-/-}$ mice also had disturbed lymphoid organogenesis, thus revealing RelB's importance in the noncanonical NF-κB pathway (Paxian et al., 2002; Yilmaz et al., 2003). *Relb*$^{-/-}$ B cells were found to have proliferative defects, but to be capable of normal IgM production and class switching (Snapper et al., 1996). In c-Rel deficient mice, both B and T cells responded poorly to mitogenic stimuli (Kontgen et al., 1995).

1.6.3.3 The role of NF-κB in T cell development and maintenance

1.6.3.3.1 The tissue specific manipulation of NF-κB

From the second half of the 1990s onwards, a number of studies began to make use of a dominant negative, or super-inhibitory form of IκBα, which could not be degraded by the IKK complex. At first, this dominant negative IκBα was transfected into a variety of cell types, including primary fibroblasts, fibrosarcoma cells, and Jurkat cells (Brockman et al., 1995; Van Antwerp et al., 1996; Wang et al., 1996). These *in vitro* studies helped to confirm NF-κB's role in the protection of many cell types from TNF induced death (Van Antwerp et al., 1996; Wang et al., 1996). Shortly afterwards began the generation of transgenic mice, expressing the dominant negative IκBα under the control of tissue specific promoters (Boothby et al., 1997; Esslinger et al., 1997).

In 1994 Marth and Rajewsky reported that the Cre-loxP recombination system could be reliably used for the conditional targeting of genes *in vivo* (Gu et al., 1994). They explained that an enzyme, Cre recombinase, could be placed under a cell type specific or developmental stage specific promoter. Mice bearing this Cre transgene would then be intercrossed to mice carrying a loxP

flanked target gene. In the offspring, Cre-loxP site-dependent recombination would occur upon Cre expression, hence leading to deletion of the target gene. Such a system would prove useful in situations where a global deletion of the target gene would lead to lethality (Gu et al., 1994).

The years that followed resulted in widespread acceptance of the Cre-loxP recombination system. A large number of studies have since used the Cre-loxP system or the dominant negative IκBα transgene to investigate the role of NF-κB in specific cell types and/or at specific developmental stages. NF-κB is clearly very important to the normal functioning of the immune system, where its role has been conserved from drosophilia to mouse and man. Of note, it is a downstream target of many receptors of the immune system: antigen-receptor, TNFSF receptors, and Toll-IL-1 receptors, among others. In recent years, studies have begun to indicate a role for NF-κB signalling in T cell development and homeostasis.

Expression of the dominant negative form of IκBα under the control of the CD2 promoter/enhancer or the proximal Lck (pLck) promoter results in decreased numbers of SP thymocytes, particularly among the CD8 lineage (Boothby et al., 1997; Esslinger et al., 1997; Hettmann and Leiden, 2000). When IKK2 is deleted in T cells, by way of $CD4^{Cre}$ or $huCD2^{iCre}$, the mice reportedly have normal thymic development, but reduced numbers of peripheral T cells, especially T_{reg} cells (Schmidt-Supprian et al., 2003; Silva et al., 2014). The T cell specific, conditional knockout of IKK1 using $CD4^{Cre}$ has recently been described. Interestingly, the mice show a large reduction in both thymic and peripheral T_{reg} cells (Chen et al., 2015). However, the reduction in peripheral T_{reg} cells is not as great as in the IKK2 deficient mice (Chen et al., 2015; Schmidt-Supprian et al., 2003). The $CD4^{Cre}$ mediated deletion of NEMO results in a dramatic loss of the more mature HSA^{lo} populations of SP thymocytes and a loss of the peripheral T cell populations (Schmidt-Supprian et al., 2003). Notably, the deletion of TAK1 in T cells, using Lck^{Cre} or $CD4^{Cre}$, yielded a similar phenotype (Liu et al., 2006; Sato et al., 2006; Wan et al., 2006).

1.6.3.3.2 Activating NF-κB in T cells

Assuming that NF-κB is necessary during T cell development and homeostasis, then the question arises as to how it may be activated. As we know, activation of the NF-κB signalling pathway occurs in response to a huge number of different stimuli. Current belief is that ligation of the pre-TCR, or of the TCR and (in naïve cells) co-stimulatory receptors, provide the main way of activating NF-κB throughout the T cell's lifespan (Gerondakis et al., 2013).

Use of an NF-κB dependent luciferase reporter gene has revealed particularly high levels of NF-κB activity during the DN3 and DN4 stages of thymocyte development, correlating with expression of the pre-TCR (Voll et al., 2000). Furthermore, constitutive pre-TCR signalling has been linked to activation of NF-κB, suggesting that NF-κB may be required for beta-selection (Aifantis et al., 2001). Studies also suggest a role for NF-κB downstream of the TCR during positive and negative selection of thymocytes. Notably, mice expressing a super-inhibitory, degradation resistant form of IκBα have few CD8SP thymocytes (Hettmann and Leiden, 2000). This is believed to be due to reduced positive selection. In support of this, mice with a constitutively active form of IKK2 show enhanced selection of CD8 lineage cells (Jimi et al., 2008).

In an attempt to explain the phenotype of the TAK1 deficient mice, it was suggested that TCR and IL-7R mediated NF-κB activation could be required during the SP stage of thymocyte development (Wan et al., 2006). However, this seems unlikely, since blocking TCR or IL-7 signalling just before positive selection does not result in a similar phenotype (McCaughtry et al., 2012; Sinclair and Seddon, 2014). It is possible that a different stimulus is activating NF-κB in SP thymocytes.

There is much evidence to suggest that TCR induced activation of NF-κB is required during an immune response. *In vivo*, IKK2 deficient CD4 T cells fail to expand to weaker KLH or OVA antigens, responding only to strong, superantigen-mediated stimulation (Schmidt-Supprian et al., 2004). Mice

expressing a degradation resistant form of IκBβ have shown defects in T cell dependent immune responses, including impaired delayed type hypersensitivity (DTH) and poor generation of an antibody response (Attar et al., 1998). In a study by Tato et al., mice transgenic for a super-inhibitory form of IκBα were challenged to *Toxoplasma gondii*. Upon stimulation, the transgenic $CD4^+$ T cells showed reduced proliferation and IFN-γ production, as compared to control cells. The authors concluded that the lack of NF-κB mediated TCR and co-stimulatory signalling may account for the reduced antigen-driven expansion among the transgenic T cell population (Tato et al., 2003). Similarly, Schmidt-Supprian et al. suggested that the lack of memory and regulatory T cells in their IKK2 deficient mice could be due to defective antigen-induced T cell activation (Schmidt-Supprian et al., 2003).

It has also been postulated that NF-κB signalling is necessary to protect T and B cell precursors from artificially high levels of TNF. During the adoptive transfer of haematopoietic stem cells into lethally irradiated hosts, excessive amounts of TNF cytokine have been noted. If the haematopoietic stem cells are NF-κB deficient, then they fail to reconstitute the host, most likely due to their TNF induced death (Claudio et al., 2006; Grossmann et al., 1999; Horwitz et al., 1997; Prendes et al., 2003; Senftleben et al., 2001b). Such observations are suggestive of a role for NF-κB downstream of the TNF receptor during the development of T cell precursors. Hence, it seems that TNF could also be a candidate for NF-κB activation in T cells, although further evidence is required.

1.7 Thesis aims

Previous studies have indicated that NF-κB signalling is likely required at various stages of T cell development and homeostasis. However, there is still uncertainty surrounding the function of NF-κB, particularly within thymocytes. During the DP stage of thymocyte development, NF-κB has been suggested to promote differentiation as opposed to cell survival (Esslinger et al., 1998; Ochoa-Garay et al., 1998). However, it has been noted that an anti-apoptotic function of NF-κB during this stage may also be possible and has not yet been ruled out (Hettmann and Leiden, 2000). Notably, studies have suggested that NF-κB may be more important during the positive selection of the CD8 than the CD4 lineage (Hettmann and Leiden, 2000; Jimi et al., 2008; Mora et al., 1999). Furthermore, the role of NF-κB in negative selection signalling is controversial, with some studies showing it to be redundant (Hettmann and Leiden, 2000), but others presenting contradictory findings (Jimi et al., 2008; Mora et al., 2001).

NF-κB does seem to be important during the SP stage of development, as indicated by the phenotype of NEMO or TAK1 conditional knockout mice, which lack mature HSA^{lo} SP thymocytes. In addition, NF-κB is necessary for the upregulation of the IL-7R during the later stages of SP thymocyte development and in recent thymic emigrants (Silva et al., 2014). Although IL-7R upregulation is not required for cells to leave the thymus (Miller et al., 2014; Silva et al., 2014), it is needed for the long-term survival of T cells in the periphery (Schluns et al., 2000).

Many unanswered questions remain. It is still uncertain as to whether NF-κB signalling is required at discrete stages during a T cell's lifespan, or whether it must be constitutively activated. Furthermore, the stimuli required for NF-κB activation, especially during the SP stage of development, have not been well investigated. Finally, the precise role of NF-κB during thymocyte development, whether for differentiation or survival, is unknown.

This study aimed to investigate the role of IKK mediated NF-κB signalling in thymocyte development and homeostasis. To this aid, we used mice with a conditional deletion of both *Ikk1* and *Ikk2* early in thymocyte development.

Our broad aims were as follows:

1) To assess the impact of a complete loss of IKK subunits on T cell development and homeostasis.

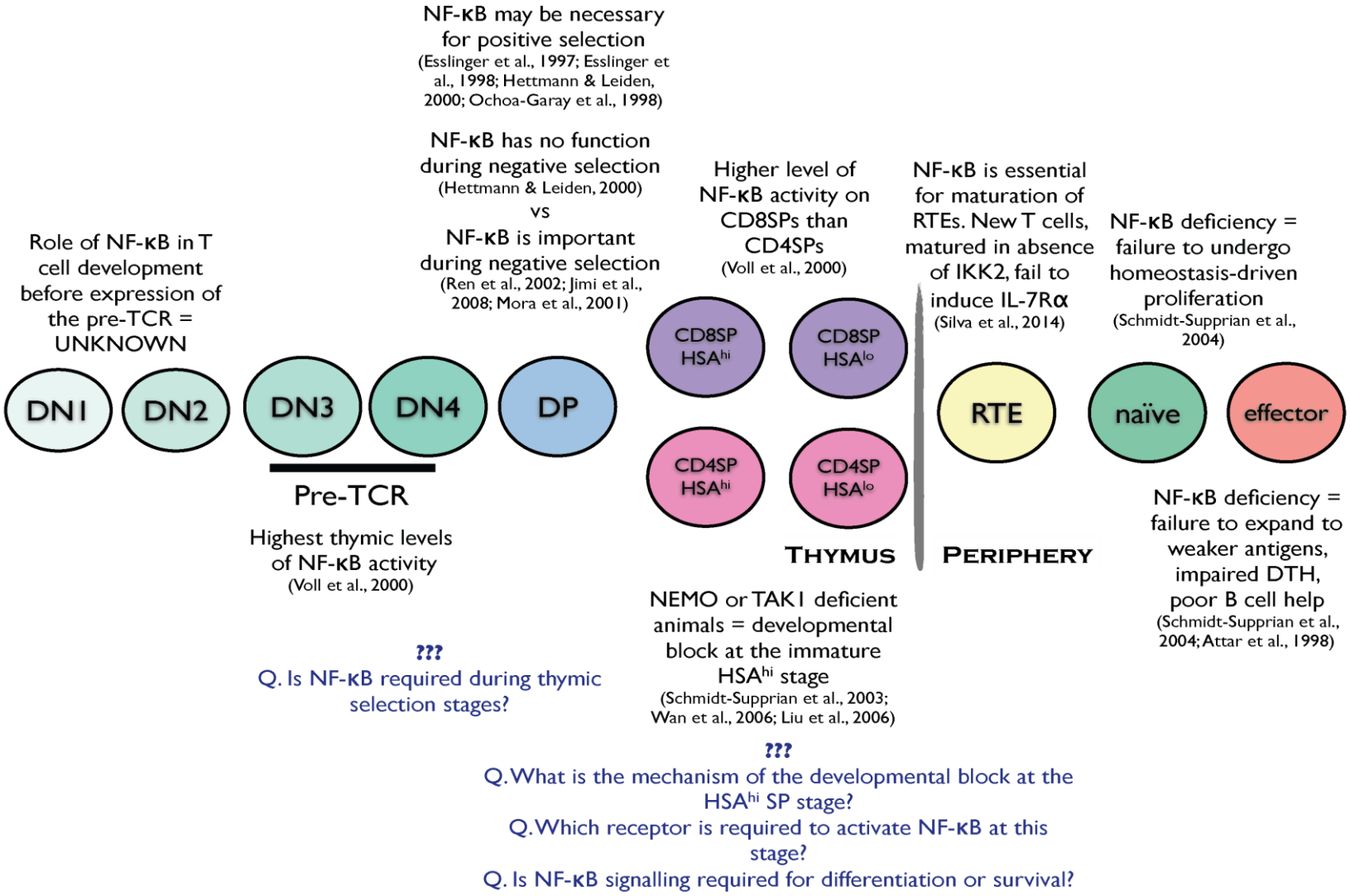
2) To investigate the nature of the stimuli activating IKK signalling during SP thymocyte development.

3) To understand the signalling pathway/s leading to IKK activation during SP thymocyte development.

Schematic 1.7 The role of NF-κB signalling during T cell development and homeostasis: current understanding and unknowns

This schematic reveals our current understanding of the role that NF-κB signalling plays during the development and maintenance of T cells. It also highlights certain gaps in our knowledge (???) that this study shall aim to address.

DTH = delayed type hypersensitivity.


NF-κB may be necessary for positive selection
(Esslinger et al., 1997; Esslinger et al., 1998; Hettmann & Leiden, 2000; Ochoa-Garay et al., 1998)
NF-κB has no function during negative selection
(Hettmann & Leiden, 2000)
vs
NF-κB is important during negative selection
(Ren et al., 2002; Jimi et al., 2008; Mora et al., 2001)
Role of NF-κB in T cell development before expression of the pre-TCR = UNKNOWN
DN1
DN2
DN3
DN4
DP
Pre-TCR
Highest thymic levels of NF-κB activity
(Voll et al., 2000)
???
Q. Is NF-κB required during thymic selection stages?
Higher level of NF-κB activity on CD8SPs than CD4SPs
(Voll et al., 2000)
CD8SP HSAhi
CD8SP HSAlo
CD4SP HSAhi
CD4SP HSAlo
THYMUS
NEMO or TAK1 deficient animals = developmental block at the immature HSAhi stage
(Schmidt-Supprian et al., 2003; Wan et al., 2006; Liu et al., 2006)
???
Q. What is the mechanism of the developmental block at the HSAhi SP stage?
Q. Which receptor is required to activate NF-κB at this stage?
Q. Is NF-κB signalling required for differentiation or survival?
NF-κB is essential for maturation of RTEs. New T cells, matured in absence of IKK2, fail to induce IL-7Rα
(Silva et al., 2014)
RTE
PERIPHERY
NF-κB deficiency = failure to undergo homeostasis-driven proliferation
(Schmidt-Supprian et al., 2004)
naïve
effector
NF-κB deficiency = failure to expand to weaker antigens, impaired DTH, poor B cell help
(Schmidt-Supprian et al., 2004; Attar et al., 1998)

Chapter 2 Materials and Methods

2.1 Mice

2.1.1 Experimental mice used in this study

Mice used in this study are listed in **Table 2.1**. All strains were housed in conventional, specific pathogen free (SPF) animal facilities at the Medical Research Council's (MRC's) National Institute for Medical Research (NIMR, London, UK). Animal procedures were performed according to strict institutional guidelines and adhered to Home Office regulations. Mice were genotyped by flow cytometric analysis of lymphocyte populations within peripheral blood, or by PCR analysis of DNA obtained from tail or ear biopsies. Genotyping was performed by Sim Tung or by Transnetyx Automated Genotyping (Cordova, Memphis, Tennessee). Transnetyx protects the intellectual rights of their screening process and thus we are not at liberty to share this information. Furthermore, the primer sequences used by Transnetyx are private and confidential and cannot be shared.

2.1.2 Generation of IKK floxed mice

The Cre-loxP recombination system was used in this study to control expression of the IKK genes. The floxed *Ikk1* and *Ikk2* alleles used were first generated in the Pasparakis and Karin laboratories, respectively (Gareus et al., 2007; Li et al., 2003). LoxP sites surrounded exons 6-8 of the *Ikk1* gene and exon 3 of the *Ikk2* gene (Gareus et al., 2007; Li et al., 2003). The mice used in our study had an additional EYFP (enhanced yellow fluorescent protein) reporter gene inserted into the Rosa26 locus and preceded by a loxP flanked strong transcriptional termination sequence (tpA) (Srinivas et al., 2001).

Mice expressing the floxed alleles were intercrossed with mice expressing either codon-improved Cre recombinase (iCre) under the control of the human

(hu) CD2 promoter (de Boer et al., 2003) or Cre recombinase under the control of the CD4 promoter (Lee et al., 2001). (The codon usage of iCre is optimised for eukaryotes (Shimshek et al., 2002). Furthermore, the iCre gene has a much reduced frequency of CpG dinucleotides as compared to the Cre gene, making it more desirable for use in mammalian cells (Shimshek et al., 2002).) Our breeding strategy resulted in a variety of different mouse strains, hetero or homozygous for floxed *Ikk1* and/or *Ikk2* alleles and expressing one of the two aforementioned Cre recombinases. *Ikk* floxed, Cre negative (Cre-) animals were also generated and served as “wild-type” (WT) controls.

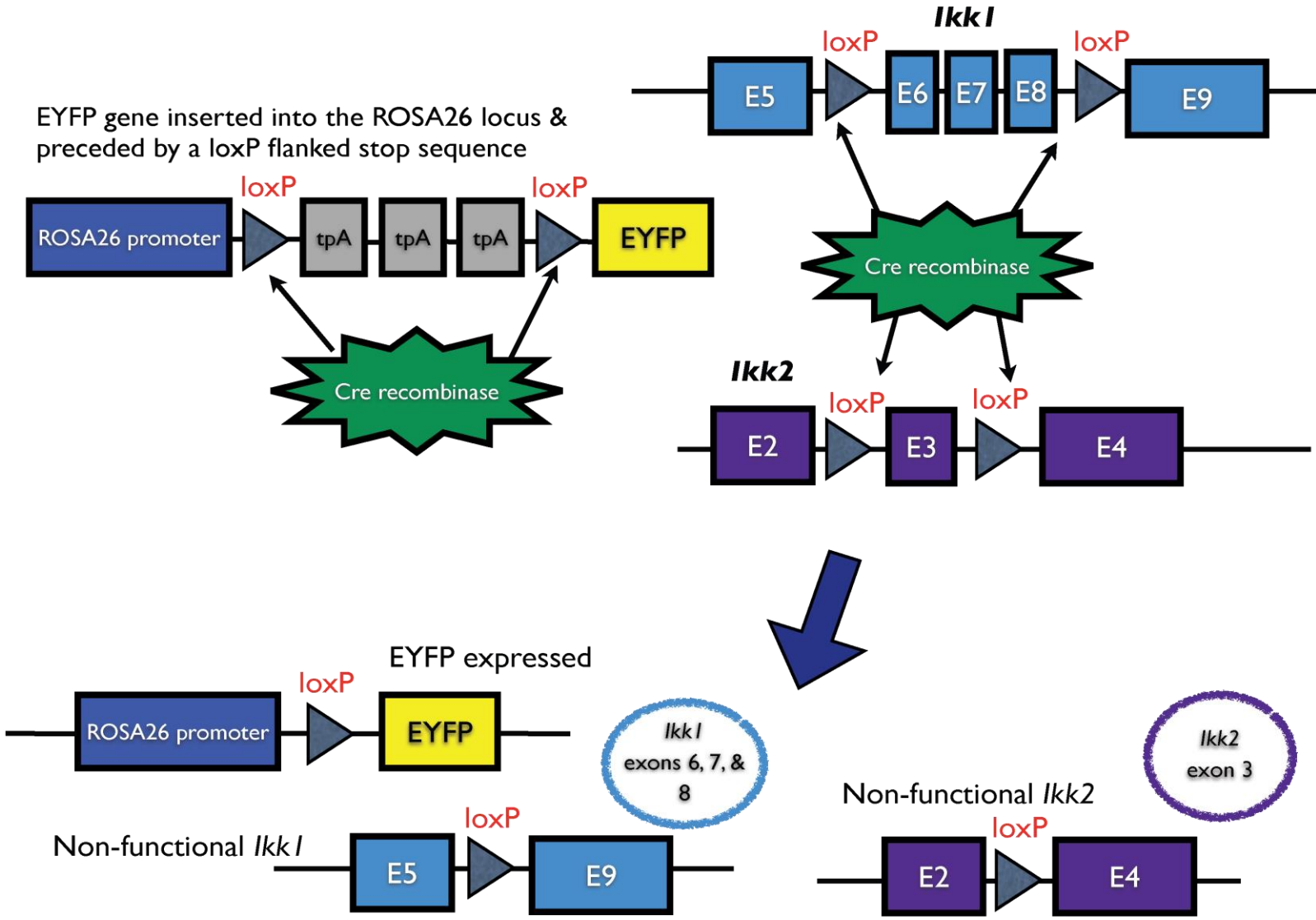
Table 2.1 Mice used in this study

Mouse strain	References
C57BL/6 (CD45.2/Ly5.2)	
C57BL/6 (CD45.1/Ly5.1)	
F5 *Rag1*$^{-/-}$ (CD45.2/Ly5.2)	(Mamalaki et al., 1993)
C57BL/6 *Rag1*$^{-/-}$	(Mombaerts et al., 1992)
huCD2^{iCre} *Ikk1*$^{fx/fx}$ R26R^{EYFP}	Generated in house
huCD2^{iCre} *Ikk2*$^{fx/fx}$ R26R^{EYFP}	Generated in house (Silva et al., 2014)
huCD2^{iCre} *Ikk1*$^{fx/fx}$ *Ikk2*$^{fx/fx}$ R26R^{EYFP} (IKKΔT^{CD2})	Generated in house
CD4^{Cre} *Ikk1*$^{fx/fx}$ *Ikk2*$^{fx/fx}$ R26R^{EYFP} (IKKΔT^{CD4})	Generated in house
CD4^{Cre} *Rela*$^{fx/fx}$ R26R^{EYFP}	Generated in house
Nfkb1$^{-/-}$	(Sha et al., 1995)
CD4^{Cre} *Rela*$^{fx/fx}$ *Nfkb1*$^{-/-}$ R26R^{EYFP}	Generated in house
Tnfrsf1a$^{-/-}$	(Rothe et al., 1993)
Tnfrsf1a$^{-/-}$ CD4^{Cre} *Ikk1*$^{fx/fx}$ *Ikk2*$^{fx/fx}$ R26R^{EYFP} (*Tnfrsf1a*$^{-/-}$ IKKΔT^{CD4})	Generated in house

Schematic 2.1 Cre-loxP recombination

This schematic demonstrates how the Cre-loxP recombination system controls expression of the genes for IKK1 and IKK2. LoxP sites surround exons 6-8 of the *Ikk1* gene and exon 3 of the *Ikk2* gene (Gareus et al., 2007; Li et al., 2003). An EYFP (enhanced yellow fluorescent protein) reporter gene is present within the Rosa26 locus and preceded by a loxP flanked stop sequence (Srinivas et al., 2001). Mice expressing the floxed alleles were intercrossed with mice expressing codon-improved Cre recombinase (iCre) under the control of the human (hu) CD2 promoter (de Boer et al., 2003) or Cre recombinase under the control of the CD4 promoter (Lee et al., 2001). huCD2^{iCre} first becomes active at the DN2 stage of T cell development, whilst CD4^{Cre} first becomes active at the DP stage of T cell development. When activated, the Cre recombinase deletes the floxed sections of the conditional *Ikk1* and *Ikk2* alleles and also deletes the stop sequence (tpA) in front of the EYFP gene (allowing expression of EYFP). EYFP expression provides a useful determinant of Cre activity.

tpA = strong transcriptional termination sequence. EYFP = enhanced yellow fluorescent protein.


EYFP gene inserted into the ROSA26 locus &
preceded by a loxP flanked stop sequence
ROSA26 promoter
loxP
tpA
tpA
tpA
loxP
EYFP
Cre recombinase
Ikk1
loxP
E5
E6
E7
E8
loxP
E9
Cre recombinase
Ikk2
loxP
E2
E3
loxP
E4
EYFP expressed
ROSA26 promoter
loxP
EYFP
Ikk1
exons 6, 7, &
8
Non-functional Ikk1
loxP
E5
E9
Ikk2
exon 3
Non-functional Ikk2
loxP
E2
E4

2.2 Media

Phosphate buffered saline (PBS)

1X PBS was made in house (NIMR's media kitchen). Dulbecco's Phosphate Buffered Saline (DPBS) was purchased (Gibco).

Fluorescence activated cell sorting (FACS) buffer

FACS buffer consisted of PBS supplemented with 0.5% (v/v) sodium azide (Sigma-Aldrich) and 0.5% (w/v) bovine serum albumin (BSA) (Sigma-Aldrich).

Handling media

Handling media consisted of air-buffered Iscove's Modified Dulbecco's Medium (AB-IMDM) (made in house) supplemented with 1% (w/v) BSA.

Complete medium (for *in vitro* culture)

Complete medium consisted of Roswell Park Memorial Institute 1640 (RPMI 1640) medium (Gibco) supplemented with 10% (v/v) fetal calf serum (FCS) (Biosera), 50μM β-mercaptoethanol (Sigma-Aldrich), and 1.5% (v/v) L-glutamine penicillin-streptomycin (PSG) solution (Sigma-Aldrich).

Ammonium-Chloride-Potassium (ACK) lysis buffer (for lysis of red blood cells)

ACK lysis buffer consisted of distilled H_2O (dH_2O) with 150mM NH_4Cl, 10mM $KHCO_3$, and 0.1mM ethylenediaminetetraaceticacid (EDTA).

2.3 Preparation of single cell suspensions

2.3.1 Thymus, spleen, and lymph nodes

The thymus, spleen, and lymph nodes (cervical, auxiliary, brachial, inguinal, and mesenteric) were dissected from mice of interest before being pressed through 75μm nylon mesh to produce single cell suspensions. Cells were then washed in handling media and pelleted by centrifugation (300g for 4min at 4^{o}C). Cells were re-suspended in handling media and cell concentrations were then obtained through use of a Scharfe Instruments CASY Counter (Scharfe System, Germany). Cells were kept on ice throughout.

2.3.2 Peripheral blood

The lateral tail veins of experimental mice were punctured with needles and droplets of blood were drawn into sodium heparinised haematocrit capillary tubes (Globe Scientific). Approximately 100μL of blood was collected from each mouse. 2mL of ACK lysis buffer was added to the samples to lyse the red blood cells. Samples were incubated in the ACK buffer for 3min at room temperature, during which they were vortexed periodically. Samples were then washed in ice-cold FACS buffer and pelleted by centrifugation (300g for 4min at 4^{o}C).

2.4 Flow cytometry

2.4.1 Surface staining with primary antibodies

For each sample, between 1 x 10^6 and 5 x 10^6 cells were stained with saturating concentrations of antibodies (Abs) (**Table 2.2**) in 100µl of FACS buffer. Samples were incubated in the dark with the antibody preparations for at least 45min at 4^{o}C. Following surface staining, samples were washed in FACS buffer, centrifuged (300g for 4min at 4^{o}C) and re-suspended. Samples were then: analysed/sorted immediately by flow cytometry (section **2.4.4**; section **2.4.5**), subjected to a secondary staining step (section **2.4.3**), or fixed with 300µL IC fixation buffer (eBioscience). Fixation, if required, took place over 15min at room temperature or 30min at 4^{o}C. Following fixation, cells were washed, centrifuged (300g for 4min at 4^{o}C), and resuspended in 100-300µL FACS buffer.

2.4.2 Intracellular staining for RIPK1

Cells were surface stained as described in section **2.4.1**. Samples were then fixed with 300µL IC fixation buffer for 30min at 4^{o}C. Following fixation, samples were washed twice in PBS, before being permeabilised with a 0.1% (v/v) solution of nonidet P-40 (NP40) (Igepal ca-630) (Sigma-Aldrich) in PBS for 3min at room temperature. Cells were then washed before being stained with anti-RIPK1 antibody or isotype control for 1hr at 4^{o}C. Following this, samples were washed twice before being stained with an anti-rabbit secondary antibody (section **2.4.3**).

Table 2.2 Primary FACS antibodies used in this study

Specificity & conjugate	Manufacturer, clone	Working dilution	Final concentration (µg/mL)
B220 eF450	eBioscience, RA3-6B2	1/200	1
CD11b PE Cy7	eBioscience, M1/70	1/400	0.5
CD11c PE	eBioscience, N418	1/200	1
CD19 APC	eBioscience, eBio1D3 (1D3)	1/100	2
CD19 PE Cy5	eBioscience, eBio1D3 (1D3)	1/400	0.5
CD2 PE	BD Pharmingen, RM2-5	1/800	0.25
CD23 PE	eBioscience, B3B4	1/300	0.67
CD25 APC	eBioscience, PC61.5	1/400	0.5
CD25 FITC	BD Pharmingen, 3C7	1/400	1.25
CD25 PE Cy7	eBioscience, PC61.5	1/400	0.5
CD4 APC	eBioscience, RM4-5	1/400	0.5
CD4 Bio	eBioscience, RM4-5	1/400	1.25
CD4-eF450	eBioscience, RM4-5	1/400	0.5
CD4 PE TR	Invitrogen, RM4-5	1/200	1
CD4 PO	Invitrogen, RM4-5	1/200	0.5
CD44 APC	eBioscience, IM7	1/400	0.5
CD44 APC-eF780	eBioscience, IM7	1/400	0.5
CD45.1 APC	eBioscience, A20	1/400	0.5
CD45.1 Bio	eBioscience, A20	1/200	2.5
CD45.1 FITC	eBioscience, A20	1/400	1.25
CD45.1 PE	eBioscience, A20	1/400	0.5
CD45.2 Bio	eBioscience, 104	1/400	1.25
CD45RB APC	eBioscience, C363.16A	1/400	0.5
CD5 APC	eBioscience, 53-7.3	1/400	0.5
CD5 FITC	eBioscience, 53-7.3	1/400	1.25

CD5 PE Cy7	eBioscience, 53-7.3	1/400	0.5
CD8α eF450	eBioscience, 53-6.7	1/400	0.5
CD8α PE Cy7	eBioscience, 53-6.7	1/400	0.5
CD8α PO	Invitrogen, 5H10	1/200	1
CD93 APC	eBioscience, AA4.1	1/100	2
HSA (CD24) APC	eBioscience, M1/69	1/400	0.5
HSA (CD24) FITC	eBioscience, M1/69	1/400	1.25
HSA (CD24) PE Cy7	eBioscience, M1/69	1/800	0.25
IgD Bio	eBioscience, 11-26c	1/200	2.5
IgM PE Cy7	eBioscience, II/41	1/200	1
IL-7R (CD127) PE	eBioscience, A7R34	1/200	1
Isotype control for RIPK1	Cell Signaling Technology	1/50	Not disclosed
MHC II eF450	eBioscience, AF6-120.1	1/200	1
Qa2 Bio	BD Pharmingen, 1-1-2	1/200	2.5
RIPK1 unconjugated	Cell Signaling Technology	1/50	Not disclosed
TCRβ APC	eBioscience, H57-597	1/200	1
TCRβ Bio	eBioscience, H57-597	1/200	2.5
TCRβ PE Cy5	eBioscience, H57-597	1/400	0.5
TCRβ PerCP Cy5.5	eBioscience, H57-597	1/400	0.5

APC = Allophycocyanin; APC-eF780 = Allophycocyanin e-Fluor 780; Bio = Biotin; eF450 = e-Fluor 450; FITC = Fluorescein isothiocyanate; PE = Phycoerythrin; PE Cy5 = Phycoerythrin cyanine 5; PE Cy7 = Phycoerythrin cyanine 7; PE TR = Phycoerythrin Texas red; PerCP Cy5.5 = Peridinin-chlorophyll protein cyanine 5.5; PO = Pacific orange.

2.4.3 Secondary staining

Where surface staining involved the use of a biotin conjugated primary antibody, then a secondary staining step was employed. Biotinylated antibodies were detected with streptavidin (SAV) APC (eBioscience) or SAV APC-eF780 (eBioscience). The working dilution for the SAV fluorochrome conjugates was 1/200 and the final concentration used was 1µg/mL. When intracellular staining for RIPK1 was performed, then a secondary detection step was also required. This involved the use of anti-rabbit Alexa Fluor 674 (Cell Signalling Technology). The working dilution for the anti-rabbit secondary Ab was 1/1000.

Cells were stained with the SAV conjugates or the anti-rabbit secondary Ab in the dark for 45min at 4^{o}C, before being washed, centrifuged (300g for 4min at 4^{o}C), and re-suspended in 100-300µL FACS buffer. RIPK1 stained samples were analysed immediately (section **2.4.4**). Biotin:SAV stained samples were either analysed immediately or fixed as described in section **2.4.1**.

2.4.4 FACS analysis of stained samples

Prepared samples were run on a BD FACSCANTO II (Beckton Dickinson, BD) or BD LSRFortessa X-20 (BD) flow cytometer with colour compensations and data analysis being performed with FlowJo V9.6.2 software (TreeStar). FACS plots shown throughout this study use log and biexponential displays.

2.4.5 Cell sorting

Prior to cell sorting, cells were stained as described in section **2.4.1**, but with handling media as opposed to FACS buffer. Following surface staining, samples were re-suspended in phenol red free AB-IMDM (made in house) or phenol red free RPMI 1640 (Gibco). Immediately after staining, sorting was carried out using a BD FACSAria (BD) or BD Influx (BD). Sorted cells were collected in handling media. Cell sorting was performed by members of the NIMR's Flow Cytometry Team.

2.5 Generation of mixed bone marrow chimeras

2.5.1 Extraction and preparation of donor bone marrow

Bone marrow was extracted from the femora and tibiae of suitable mouse strains, disrupted, and resuspended in DPBS. Mature T cells were then depleted from the cell suspension as follows. TCR biotinylated antibody was added to the cell suspension and left to stain for 15min on ice. Streptavidin conjugated Dynabeads (Invitrogen) were then added, and the mixture rotated for 20min at 4^{o}C. The Dynabeads:antibody:TCR conjugates were then removed from the cell suspension through use of the Dyna magnet (Invitrogen). The remaining bone marrow cells were washed, centrifuged (300g for 8min at 4^{o}C), and resuspended in handling media to a final concentration of between 32 and 40 million cells per mL.

2.5.2 Irradiation of host mice and reconstitution with donor bone marrow

C57BL/6 *Rag1*$^{-/-}$ host mice were sub-lethally irradiated with 500 rads (caesium source) and then treated with Baytril (0.02% (v/v) in H_2O) for one month to prevent opportunistic infection. Approximately 24hr after irradiation, their lateral tail veins were injected with 4-5 million of the prepared bone marrow cells. Mice were then left for a minimum of 8 weeks to allow full reconstitution of the myeloid and lymphoid compartments. Immune system reconstitution was confirmed by phenotypic analysis of peripheral blood.

2.6 Intraperitoneal injections of blocking antibody

2.6.1 *In vivo* blockade of TNF

A blocking antibody towards murine TNF (BioXcell, BE0058) was administered to animals of interest via intraperitoneal (I.P.) injection. Each injection contained 1mg of antibody. Two different regimes were employed. The first took place over a one-week period and involved 3 injections in total (days 0, 2, and 4), before sacrificing the animal on day 7. The second took place over two weeks and involved 6 injections (days 0, 2, 4, 7, 9, and 11), before sacrifice on day 14. For control animals, the same protocols were followed, but PBS used in place of antibody.

2.6.2 *In vivo* blockade of CD27L

A blocking antibody towards murine CD27L (BioXcell, BE0022) was administered to animals of interest via intraperitoneal (I.P.) injection. Each injection contained 1mg of antibody. A total of 3 injections were administered (days 0, 2, and 4), before the animal was sacrificed on day 7. For control animals, the same protocol was followed, but PBS used in place of antibody.

2.7 Cell culturing

2.7.1 Culture conditions

Whole thymus was mashed and liberated cells resuspended in complete medium. Cells were then plated in a 24 well plate at a concentration of 1-2.5 million per mL of media. The thymocytes were cultured with a variety of ligands, blocking Abs, or inhibitors (**Table 2.3**) at 37^{o}C with 5% CO_2. Sterile technique was practiced throughout. At the end of the culture period, reactions were stopped, and cells were washed and stained for later analysis by flow cytometry, as described in section **2.4**.

2.7.2 Assessing cell death induction

Cell viability was examined through use of the Live/Dead Fixable Dead Cell Stains (Invitrogen). These are fluorescent dyes, which react with the amine groups on the outside and inside of cells. If a cell is dead, the dye can penetrate the cell membrane, resulting in very bright fluorescence. The Live/Dead Fixable Near-IR stain (Invitrogen, L10119) or the Live/Dead Fixable Yellow stain (Invitrogen, L34959) was used as appropriate. Both stains were used at a working dilution of 1/1000. If used, the Live/Dead stain was added to the cells during the surface staining step described in section **2.4.1**. (If a Live/Dead stain was to be used, then it and all antibodies were diluted in PBS, as opposed to FACS buffer containing BSA. This was to prevent the Live/Dead dye reacting with the amine groups within the BSA.)

For analysis of active caspase-8, the CaspGLOW Red Active Caspase-8 Staining Kit (BioVision Incorporated, K198-100) was used, with staining carried out according to the kit instructions. Briefly, cultured cells (between 1 x 10^6 and 4 x 10^6) were washed, pelleted, and re-suspended in 300µL of PBS. 2µL of Red-IETD-FMK was then added, and the cells cultured for 1hr at 37^{o}C with 5% CO_2. Sterile technique was practiced for the duration of the caspase-8 staining

period. Cells were then washed twice in wash buffer (BioVision Incorportated), before surface staining was carried out as described in section **2.4.1**.

Table 2.3 Ligands, antibodies, or inhibitors used during cell culture

Ligand/Ab/Inhibitor added to culture	Concentrations used	Source
Ligand		
APRIL (TNFSF13)	100ng/mL	R&D Systems, cat. no. 7907-AP-010
BAFF (TNFSF13B)	100ng/mL	R&D Systems, cat. no. 2106-BF-010
CD27L (TNFSF7)	0.41 to 100ng/mL	R&D Systems, cat. no. 783-CL-050
GITRL (TNFSF18)	100ng/mL	R&D Systems, cat. no. 2177-GL
IL-7	10ng/mL	Peprotech, cat. no. 217-17
LIGHT (TNFSF14)	100ng/mL	R&D Systems, cat. no. 1794-LT-025
TLA1 (TNFSF15)	100ng/mL	R&D Systems, cat. no. 1896-TL-010
TNF (TNFSF2)	0.1 to 1000ng/mL	Peprotech, cat. no. 315-01A
TRAIL (TNFSF10)	100ng/mL	R&D Systems, cat. no. 1121-TL-010
Blocking Ab		
Anti-TNFR1 (CD120a)	0.78 to 200µg/mL	Ebioscience, clone 55R-170
Inhibitor		
IKK2 inhibitor BI605906	10µM	A gift from Philip Cohen, University of Dundee
Birinapant	11.7 to 200µM	Selleck Chemicals, cat. no. S7015
GDC-0152	11.7 to 200µM	Selleck Chemicals, cat. no. S7010
Necrostatin-1	0.05 to 100µM	Santa Cruz Biotechnology, cat. no. sc-200142
Z-IETD-FMK (caspase-8 inhibitor)	20µM & 40µM	BD, cat. no. 550380
Z-FA-FMK (negative control for caspase inhibitors)	20µM & 40µM	BD, cat. no. 550411

2.8 RNA sequencing

2.8.1 RNA isolation

Thymocytes were sorted as described in section **2.4.4**. The purified cell populations were then washed and pelleted. RNA was extracted from the cells using the Isolate II RNA mini kit (Bioline), following the kit instructions. Briefly, the cell pellets were lysed in the presence of guanidinium thiocyanate to deactivate endogenous RNases. Ethanol was added and the sample then passed through a column containing a silica membrane to which the RNA bound. Genomic DNA was removed by an on-column DNase I digestion. The sample was washed of impurities (salts, metabolites, and cellular components), and the RNA then eluted in RNase-free water. The quality of the extracted RNA was checked using a NanoDrop spectrophotometer and an Agilent Bioanalyzer.

2.8.2 Preparation of a cDNA library and cluster generation on the Illumina high throughput sequencer

The Illumina Truseq RNA Library Preparation kit (Illumina) was used to prepare cDNA libraries for sequencing. Manufacturer's instructions were followed. Briefly, Poly-T oligo-attached magnetic beads were used to extract the poly-A containing mRNA from 0.5µg of total RNA. The mRNA was broken into small fragments using divalent cations and a high temperature. Reverse transcriptase and random primers were used to produce first strand cDNA from the fragmented mRNA. DNA Polymerase I and RNase H then enabled synthesis of second strand cDNA. Following end repair and addition of a single "A" base, the cDNA fragments were ligated to adapters. PCR was used to increase the number of cDNA fragments that had adapters ligated to both ends. The quality of the cDNA libraries was checked using an Agilent Bioanalyzer. Cluster generation on the Illumina Genome Analyser IIx was then performed.

Cluster generation on the Illumina high throughput sequencer was always performed by members of the NIMR's High-Throughput Sequencing Team.

cDNA libraries were prepared by the NIMR's High-Throughput Sequencing Team, Charles Sinclair, or by myself.

2.8.3 Analysis of sequencing data

The read sequences obtained from the Illumina high throughput sequencer were analysed using Strand NGS software (Strand Genomics, Inc.). First, the reads were aligned to build version mm9 of the Mus musculus genome. Then the aligned reads were normalised using DEseq (Anders and Huber, 2010), before being presented as number of reads per kilobase of exon per million sequenced reads (nRPKM) (Mortazavi et al., 2008).

Note: 3 independent RNA sequencing experiments are referred to in this study. All 3 experiments required a pure population of cells to be sorted prior to RNA extraction. Each RNA sequencing experiment made use of either 3 or 4 independent cell sorts and RNA extraction procedures. However, for each RNA sequencing experiment, cluster generation on the Illumina high throughput sequencer was performed only once.

2.9 Data presentation and statistical tests

2.9.1 Calculation of absolute cell numbers

Data for the calculation of absolute cell numbers was obtained from a Scharfe Instruments CASY Counter and from the BD FACSCANTO II or BD LSRFortessa X-20. Calculations for absolute cell numbers were performed in Microsoft Excel for Mac V14.5.3 (Microsoft Corporation) and R V2.14.1 (The R Foundation for Statistical Computing).

Absolute cell numbers were calculated as follows:
Total single live cell number (obtained from the CASY counter) x Frequency of total single live lymphocytes x Frequency of the cell population of interest

R V2.14.1 was used to create and execute a code for the calculation of absolute cell numbers. This made the process more efficient and less error-prone. The code was created by Iren Bains.

2.9.2 Graphs

Bar charts and line graphs were produced using Prism 6 for Mac OS X (Graphpad Software, Inc.). FACS density plots and FACS histograms were produced using FlowJo V9.6.2. The Student's t-test was used to identify statistical differences between groups. Statistical calculations were performed in Microsoft Excel for Mac V14.5.3 and Prism 6 for Mac OS X.

Chapter 3 The role of NF-κB signalling during T cell development

3.1 Introduction

The strength and duration of TCR signalling is important for selection of thymocytes (Hogquist, 2001). During thymocyte development, TCR signalling activates many transcription factors, including NF-κB. However, it is unclear what role NF-κB plays in thymic maturation (Voll et al., 2000). Interestingly, the level of NF-κB signalling has been shown to change during the DN stages of thymocyte development (Gerondakis et al., 2013). The use of an NF-κB dependent luciferase reporter gene found expression to be highest during DN3 and DN4 – the same stages during which the pre-TCR is expressed (Voll et al., 2000). Numbers of DN4 thymocytes were reduced in mice with a super-inhibitory form of IκBα (IκBα super-repressor), but increased in mice expressing a constitutively active form of IKK2 (Voll et al., 2000). Furthermore, the proliferative burst that occurs between late DN3 and DN4 was found to be decreased in the absence of NF-κB signalling (Aifantis et al., 2001). From such data, it seems that NF-κB plays a role in beta selection early in T cell development.

The role of NF-κB signalling during positive and negative selection is somewhat controversial. Most studies investigating the role of NF-κB in selection have made use of mice expressing transgenes for a non-degradable, super-inhibitory form of the IκBs and also for TCRs that recognise self-peptides with various avidities. When TCR transgenes with an intermediate avidity for self-peptide:MHC are present, NF-κB has been said to play a role in positive selection (Hettmann and Leiden, 2000; Mora et al., 2001) or in positive selection of CD8, but not CD4 thymocytes (Jimi et al., 2008). It has been suggested that the higher expression levels of the anti-apoptotic protein Bcl-2 during positive selection of CD4 thymocytes mean that NF-κB induced survival signals are not necessary (Gerondakis et al., 2013). The effect of NF-κB

signalling on negative selection has been studied in mice expressing TCR transgenes with a high avidity for self-peptide:MHC in combination with IκB super-repressors. Whilst some studies found NF-κB to be important for negative selection (Mora et al., 2001), other studies concluded that it played no role (Hettmann and Leiden, 2000). As with positive selection, it has also been suggested that NF-κB signalling is important for the negative selection of $CD8^+$, but not $CD4^+$ cells (Jimi et al., 2008).

Activation of NF-κB signalling relies on the formation of a functional IKK complex. Loss of either NEMO or of both IKK1 and IKK2 together has been shown to cause a complete block in canonical NF-κB activation (Li et al., 2000; Rudolph et al., 2000). IKK1's main NF-κB dependent role involves activation of the non-canonical pathway in response to lymphotoxin-β (LTβ). LT signalling is required for lymphoid organ development, and for this reason IKK1 is often cited as being important for the control of adaptive immunity. However, it remains unclear as to whether there is a T cell intrinsic requirement of IKK1 for normal T cell homeostasis. Through the use of *Ikk1*$^{-/-}$ bone marrow chimeras, IKK1 was found to be necessary for the normal development of mature B cells. However, such chimeras were found to contain normal numbers of total thymocytes and peripheral T cells (Kaisho et al., 2001; Senftleben et al., 2001a). Mice expressing a dominant negative form of IKK1, specific to the T cell lineage, have been described. They were found to have normal T cell development and function (Ren et al., 2002). Such data suggests that IKK1 is not necessary for normal T cell homeostasis. More recently, mice with a conditional deletion of IKK1 in $CD4^+$ cells were described (Chen et al., 2015). These mice were shown to have a significant reduction of T_{reg} cells in the thymus and periphery. CD4 cells from these mice also showed poor lymphopenia induced expansion in *Rag1*$^{-/-}$ hosts and reduced responses to *in vitro* stimuli (Chen et al., 2015). Hence, there may indeed be a T cell intrinsic requirement for IKK1.

The T cell specific deletion of IKK2 has been described (Schmidt-Supprian et al., 2003; Silva et al., 2014), as have mice in which endogenous IKK2 was replaced with a kinase dead IKK2 mutant (Ren et al., 2002; Schmidt-Supprian

et al., 2003). In one study, the kinase dead form of IKK2 was found to have no effect on T cell development. However, the analysis was somewhat superficial, since absolute T cell numbers were not calculated (Ren et al., 2002). A separate study found that mice carrying the kinase dead form of IKK2 had a 50% reduction in CD8SP thymocytes and scarcely any peripheral T cells (Schmidt-Supprian et al., 2003). Mice with a conditional deletion of IKK2 exhibit normal thymic development (Schmidt-Supprian et al., 2003; Silva et al., 2014). Whilst some studies report only reductions in the size of regulatory and memory T cell populations in the periphery of these mice (Schmidt-Supprian et al., 2003), others find an additional reduction in naïve cells (Silva et al., 2014).

The conditional deletion of NEMO in T cells using $CD4^{Cre}$ has been described by Schmidt-Supprian et al. (Schmidt-Supprian et al., 2003). Although total thymocyte numbers were apparently normal, there was a profound reduction in the CD8SP population and a small reduction in CD4SPs. In both subsets, mature HSA^{lo} thymocytes were almost completely absent, and the periphery had scarcely any T cells (Schmidt-Supprian et al., 2003).

Transcriptional activity of NF-κB is mediated by dimers of Rel family proteins, of which there are five members: RelA, RelB, c-Rel, NF-κB1, and NF-κB2. The NF-κB1 and NF-κB2 genes encode for the large protein precursors p105 and p100, respectively. These large proteins are ubiquitinated and eventually degraded by the proteasome, allowing p105 to become p50, and p100 to become p52. In its most common form, the NF-κB dimer is composed of p50 and RelA (Ghosh et al., 1998). Many studies involving mice lacking one or more Rel family members have not shown any gross defect in lymphocyte development, thus arguing redundancy or overlapping functions between members. *Rela*$^{-/-}$ mice were shown to be embryonic lethal (dying at gestation day 15-16) due to massive apoptosis of hepatocytes (Beg et al., 1995). However, fetal liver cells taken from the *Rela*$^{-/-}$ mice were able to populate host mice and give rise to a T cell repertoire. One such study described formation of a completely normal T cell compartment in the hosts following reconstitution with *Rela*$^{-/-}$ fetal liver (Beg et al., 1995), whilst another found the hosts to have around 2.5 fold fewer thymic T_{reg} cells than controls (Isomura et al., 2009).

Disruption of the RelB gene causes loss of dendritic cells, but does not affect T or B cell development (Burkly et al., 1995). T cell development in c-Rel knockout mice appears largely normal. However, T_{reg} cell numbers are reduced and c-Rel deficient T cells are less able to produce IL-2, thereby showing impaired proliferative ability (Isomura et al., 2009; Kontgen et al., 1995).

Nfkb1$^{-/-}$ mice lack both the large inhibitory protein p105 and also the mature p50 subunit. Initially, *Nfkb1*$^{-/-}$ mice were described as having no abnormality in T cell number or subsets (Sha et al., 1995). However, a more recent study found the NF-κB1 family member to be very important for the regulation of CD8 T cell development in the thymus (Gugasyan et al., 2012). In *Nfkb1*$^{-/-}$ mice a population of CD8SP thymocytes with memory like properties ($CD44^{hi}CD24^{lo}$) developed and survived to populate the periphery. This phenotype was attributed to defects in positive and negative selection. Normal numbers of DN and DP thymocytes suggested that early T cell development was not affected (Gugasyan et al., 2012). Serines 927 and 933 of NF-κB1 are important phosphorylation sites that target the protein for degradation. Mice expressing a mutant *Nfkb1*$^{S927A,\ S932A}$ knock-in gene produce a proteolytically resistant form of p105, which contains alanine instead of serine residues at positions 927 and 933 (Sriskantharajah et al., 2008). This p105 can undergo constitutive processing to p50, but is not degraded by the proteasome and therefore continues to mediate NF-κB inhibitory functions. The thymus of *Nfkb1*$^{S927A,\ S932A}$ mice revealed low numbers of T_{reg} cells and the spleen showed reduced T_{reg} cells and $CD4^+$ memory cells. $CD4^+$ T cells from the mutant mice were found to have less NF-κB complex formation in response to anti-CD3 and anti-CD28 stimulation. The authors concluded that, in $CD4^+$ T cells, TCR mediated NF-κB activation is reliant on the correct processing of p105 (Sriskantharajah et al., 2008). Other studies have reported normal thymocyte populations and T_{reg} numbers in *Nfkb1*$^{-/-}$ mice (Isomura et al., 2009). This suggests that the inhibitory function of the mutant protein, rather than an absolute requirement for p105 expression, underlies the phenotype of the *Nfkb1*$^{S927A,\ S932A}$ mice.

Functional redundancy between Rel family members is evident in compound knockouts. Mice deficient in more than one NF-κB subunit tend to show greater

defects in T cell homeostasis. When radiation chimeras were made with bone marrow from *Nfkb1*$^{-/-}$ *Rela*$^{-/-}$ donors, the hosts reconstituted their T cell compartments poorly, but overcompensated for the defect by producing an excess of granulocytes. Surprisingly, when mixed chimeras were made (with wild-type bone marrow), NF-κB1/RelA double deficient lymphocytes did survive to populate the periphery, suggesting that the requirement for NF-κB signalling was T cell extrinsic (Horwitz et al., 1997). RelA/c-Rel double deficient hematopoietic stem cells show mild maturational defects (Grossmann et al., 1999). In contrast, cRel/p50 double deficient mice have normal production of naïve cells, but lack both memory and regulatory T cells (Zheng et al., 2003).

The cytokine IL-7 is very important for maintaining correct T cell homeostasis. Not only is it involved with T cell development, but it is also required for maintenance of naïve and regulatory populations in the periphery (Jameson, 2005). Recent evidence has suggested that tonic NF-κB signalling is required for upregulation of the IL-7R as cells leave the thymus and enter the periphery (Miller et al., 2014; Silva et al., 2014). IKK2 deficient naïve T cells have a reduction in IL-7R expression, correlating with a decrease in *Il7r* mRNA levels (Silva et al., 2014). Mice with a non-degradable form of IκBα have also shown reduced IL-7R expression on their naïve T cells (Miller et al., 2014).

The aim of this Chapter was to investigate the role of NF-κB signalling in T cell development and maintenance of the peripheral T cell compartment. We achieved this by manipulating NF-κB signalling at the level of both the IKK complex and expression of Rel family members. Specifically, we examined the following:

- The cell intrinsic role of IKK1 in T cells.
- The impact of a complete loss of IKK subunits on T cell homeostasis.
- The role of specific Rel family members in T cell development.

3.2 Results

3.2.1 Reduced thymic T_{reg} cells in huCD2^{iCre} *Ikk1*$^{fx/fx}$ R26R^{EYFP} mice

We first wanted to understand the role of IKK1 in T cell development. In order to do this we bred mice with conditional alleles of IKK1 (*Ikk1*$^{fx/fx}$) (Gareus et al., 2007), which expressed the codon-improved Cre recombinase (iCre) (Shimshek et al., 2002) under the control of the human CD2 (huCD2) promoter and locus control region (de Boer et al., 2003). The mice generated also included a Cre reporter enhanced yellow fluorescent protein (EYFP) construct that had been targeted to the Rosa26 locus (R26R^{EYFP} reporter mice, floxed stop sequence proceeding the EYFP gene), thus enabling a read-out of Cre activity (Srinivas et al., 2001). The huCD2 locus control region and promoter has been reported to drive transgene expression only among cells of the lymphoid lineage (de Boer et al., 2003; Zhumabekov et al., 1995). Amongst T cells, huCD2^{iCre} has been found to initiate deletion of target genes from the DN2 stage of thymocyte development onwards (de Boer et al., 2003). We compared our huCD2^{iCre} *Ikk1*$^{fx/fx}$ R26R^{EYFP} (IKK1 deficient) mice with the previously described huCD2^{iCre} *Ikk2*$^{fx/fx}$ R26R^{EYFP} (IKK2 deficient) mice (Silva et al., 2014).

Analysis of the thymii of the IKK1 deficient mice revealed normal numbers of total thymocytes, DP cells, and SP cells (**Figure 3.1**). Within the SP thymocyte compartments, cells can be further characterised according to their expression of heat stable antigen (HSA, CD24). Expression of HSA is lost as SP thymocytes mature, enabling us to identify two distinct cell populations, HSA^{hi} (immature) and HSA^{lo} (mature), within the SP compartments (Tian et al., 2001). We found normal numbers of HSA^{hi} and HSA^{lo} cells in the CD4SP and CD8SP populations of IKK1 deficient mice (**Figure 3.1**). In the IKK2 deficient mice, there was a significant increase in cell numbers within the HSA^{hi} subset of both CD4SP and CD8SP populations (**Figure 3.1B**). Strikingly, within the CD4SP population, T_{reg} cells (CD25^{+}) were decreased in percentage and number in the IKK2 and, to a lesser extent, in the IKK1 deficient mice (**Figure 3.1**).

3.2.2 Reduced memory and T_{reg} cells in the periphery of huCD2^{iCre} *Ikk1*$^{fx/fx}$ R26R^{EYFP} mice

To investigate peripheral T cell homeostasis, we examined T cell populations within the spleen and lymph nodes (LNs) of IKK deficient strains. In contrast to huCD2^{iCre} *Ikk2*$^{fx/fx}$ R26R^{EYFP} mice, the huCD2^{iCre} *Ikk1*$^{fx/fx}$ R26R^{EYFP} mice had normal numbers of naïve (CD44^{lo}CD25^{-}) T cells (**Figure 3.2B**). However, both IKK1 and IKK2 deficient animals showed a clear reduction in the percentage and number of CD4^{+}CD25^{+} regulatory T cells and CD4^{+}CD44^{hi}CD25^{-} memory cells. This reduction was greater in mice lacking IKK2 (**Figure 3.2A & B**). The CD8^{+} T cell compartment appeared normal in the IKK1 deficient animals, whereas IKK2 deficiency resulted in a significant loss of both naïve and memory CD8 populations (**Figure 3.2B**). IKK2 deficient naïve T cells have previously been shown to have reduced expression of the IL-7R (Silva et al., 2014). For this reason, we investigated IL-7R levels on the naïve cells of IKK1 deficient mice. As compared to controls, IKK1 deficient CD4 naïve cells had normal expression of the IL-7R, whilst the CD8 naïve cells did show a small, but significant, reduction in surface expression (**Figure 3.2C**). There was no significant difference in total peripheral T cell numbers between the IKK1 deficient mice and the wild-type (WT) mice. However, as expected, the IKK2 deficient mice were found to have significantly fewer total T cells than the WT controls (**Figure 3.2B**).

In summary, we found that T cell homeostasis was disrupted in the IKK1 deficient mice, albeit to a lesser extent than observed in the IKK2 deficient mice. Thymic development was largely normal in the IKK1 deficient mice, although numbers of T_{reg} cells were reduced. In the periphery of the IKK1 deficient animals, naïve T cells appeared normal, but CD4^{+} memory cells and T_{reg} cells were significantly reduced.

3.2.3 Early thymocyte development is unperturbed in the absence of NF-κB activation

There is clear redundancy between IKK1 and IKK2. For this reason, we wished to investigate T cells doubly deficient in both IKK1 and IKK2 proteins, which would completely lack IKK function (referred to as IKK deficient throughout) and would therefore be unable to induce release of NF-κB dimers. We generated huCD2^{iCre} *Ikk1*$^{fx/fx}$ *Ikk2*$^{fx/fx}$ R26R^{EYFP} mice (IKKΔT^{CD2} hereon) with a Cre mediated deficiency of both IKK1 and IKK2 specific to the lymphoid lineage. huCD2^{iCre} induces deletion from the DN2 stage of thymocyte development (de Boer et al., 2003). In case there were profound blocks in DN thymocyte development, we also generated CD4^{Cre} *Ikk1*$^{fx/fx}$ *Ikk2*$^{fx/fx}$ R26R^{EYFP} mice (IKKΔT^{CD4}). In this strain, Cre recombinase is under the control of the CD4 enhancer/promoter/silencer, and hence gene deletion does not occur until the DP stage of thymocyte development (Lee et al., 2001). By comparing both strains of mice we were therefore able to assess the effect of IKK gene deletion at early and slightly later stages of thymocyte development.

Within the CD4 CD8 double negative (DN) population, CD44 and CD25 can be used to further characterise cells into developmentally sequential DN1 (CD44^{+}CD25^{-}), DN2 (CD44^{+}CD25^{+}), DN3 (CD44^{-}CD25^{+}), and DN4 (CD44^{-}CD25^{-}) subsets, as defined by Godfrey et al. (Godfrey et al., 1993). Since huCD2^{iCre} initiates deletion in the DN2 stage, we examined the number and representation of DN3 and DN4 populations in the IKKΔT^{CD2} mice. We saw no difference in percentage or number of DN3 and DN4 cells between the IKKΔT^{CD2} mice and the Cre- controls (**Figure 3.3A & C**). YFP expression confirmed Cre activation during the DN2 stage of thymocyte development onwards. About 50% of DN2 cells were YFP+. This rose to 98% by the time thymocytes reached the DN3 stage of development (**Figure 3.3B**). The fact that DN3 and DN4 thymocyte populations seem identical between Cre- and Cre+ mouse strains indicates that IKK1 and IKK2 are not required during the late double negative stages of development when the TCR is being rearranged.

This suggests that NF-κB signalling is not needed for beta selection or DN survival or expansion.

Looking within the DP population, we can further define cells at different temporal stages of selection according to their expression of CD5 and TCR, as previously described (Saini et al., 2010). DP1 cells ($TCR^{lo}CD5^{lo}$) have not yet initiated selection, DP2 cells ($TCR^{int}CD5^{hi}$) are in the early stages of selection of CD4 and CD8 lineage cells, and DP3 cells ($TCR^{hi}CD5^{int}$) are in the late stage of DP selection into the CD8 lineage (Saini et al., 2010). All DP populations (DP1-3) were found to be very similar between $IKK\Delta T^{CD2}$ mice and Cre- controls (**Figure 3.3A & D**). In confirmation of this, we also observed no difference in DP subsets in the $IKK\Delta T^{CD4}$ strain (**Figure 3.3D**). Therefore, the timing of deletion of the IKK complex did not seem to influence DP thymocyte development. Our findings suggest that IKK activity is redundant for early T cell development.

3.2.4 Maturation of SP thymocytes is blocked in the absence of NF-κB signalling

Next we assessed the potential role of NF-κB signalling in late thymocyte development using $IKK\Delta T^{CD4}$ mice. Although overall thymus size remained unchanged, analysis of SP compartments revealed profound perturbations (**Figure 3.4A, B & C**). Numbers of SP thymocytes were reduced in $IKK\Delta T^{CD4}$ mice, with the reduction being most pronounced among the CD8 lineage. The reduction in SP numbers was largely attributed to the loss of the most mature HSA^{lo} subpopulations. Interestingly, while numbers of HSA^{hi} CD4SP thymocytes appeared normal, HSA^{hi} CD8SPs were significantly reduced in number (**Figure 3.4C**). A similar developmental block was also observed in $IKK\Delta T^{CD2}$ mice (data not shown).

Enumeration of naïve phenotype $CD44^{lo}$ cells in the periphery of $IKK\Delta T^{CD4}$ mice revealed a substantial reduction in numbers of both CD4 and CD8 lineages. In contrast, memory, and regulatory T cells were detectable (**Figure 3.5A, B & D**). However, closer examination revealed these populations were largely YFP

reporter -ve, indicating that these may represent cells that had escaped Cre mediated deletion of their *Ikk* genes (**Figure 3.5C & D**). Similar analysis of naïve populations revealed evidence of such deletion escapants in these subsets too, particularly among the few CD8 naïve T cells present in the IKK deficient mice (**Figure 3.5C**).

Since absence of either IKK1 or IKK2 alone was shown to lead to a reduction in IL-7R expression on naïve, peripheral T cells, we examined IL-7R expression by the few naïve T cells present in our IKKΔT^{CD4} mice. Within the CD4 naïve population, IL-7R expression was dramatically reduced on IKK deficient cells as compared to WT controls. In contrast, IL-7R expression was only minimally reduced on the CD8 naïve IKK deficient cells (**Figure 3.5E**). However, YFP expression indicated that many of these cells may have failed to delete their *Ikk* genes (**Figure 3.5C**).

Therefore, IKK mediated NF-κB signalling is essential for normal thymic development. Our results suggest that, in the absence of NF-κB signalling, there is a block in thymocyte maturation between the HSA^{hi} and HSA^{lo} stages of development (**Figure 3.4D**), and a consequential failure to populate the peripheral T cell compartment.

3.2.5 The defects in T cell development in IKK deficient mice are cell intrinsic

Expression of Cre recombinase, when under the control of the CD4 enhancer/promoter/silencer, has been reported to be specific to T cells (Lee et al., 2001). However, since other cells (for example dendritic cells) have been known to express the CD4 antigen, it was possible that gene recombination may also occur in these cells. If so, this could mean that the defects seen in the NF-κB deficient animals were not T cell intrinsic. To exclude this, we generated competitive bone marrow chimeras using T cell depleted bone marrow from congenic CD45.1^{+} mice together with bone marrow from CD4^{Cre} *Ikk2*$^{fx/fx}$ R26R^{EYFP} mice ("CD4^{Cre} *Ikk2*$^{fx/fx}$" chimeras), IKKΔT^{CD4} mice ("CD4^{Cre} *Ikk1*$^{fx/fx}$

Ikk2$^{fx/fx}$ R26R^{EYFP}" chimeras) or Cre- littermate mice ("control" chimeras). Bone marrow was injected in a 1:1 ratio into the tail vein of irradiated *Rag1*$^{-/-}$ hosts. After successful reconstitution of the host mice, we then calculated the percentage of T cells in the thymus and periphery that were CD45.1^{-} (i.e. derived from the transgenic donors).

In the control chimeras the percentage of CD45.1^{-} cells remained similar among all T cell populations in the thymus and periphery (**Figure 3.6**). Between the control chimeras and the CD4^{Cre} *Ikk2*$^{fx/fx}$ chimeras, there was no difference between the percentage of CD45.1^{-} cells present in the DP, HSA^{hi}, and CD4SP HSA^{lo} populations. However, among the CD8 HSA^{lo} population, there was a slight reduction in the percentage of CD45.1^{-} cells (**Figure 3.6**). In the periphery of the CD4^{Cre} *Ikk2*$^{fx/fx}$ chimeras there were very few CD45.1^{-} cells. This was especially evident amongst the memory and regulatory populations (**Figure 3.6**).

The percentage of CD45.1^{-} cells within the DP and CD4SP HSA^{hi} thymocyte populations were similar between the control chimeras and the CD4^{Cre} *Ikk1*$^{fx/fx}$ *Ikk2*$^{fx/fx}$ R26R^{EYFP} chimeras (**Figure 3.6**). This indicates that there is no selective disadvantage in having a block in NF-κB signalling among these cell types. This data agrees with the phenotype of the intact IKKΔT^{CD4} (CD4^{Cre} *Ikk1*$^{fx/fx}$ *Ikk2*$^{fx/fx}$ R26R^{EYFP}) mouse, whereby the DP and CD4SP HSA^{hi} populations appeared normal (**Figure 3.4**). Within the CD4^{Cre} *Ikk1*$^{fx/fx}$ *Ikk2*$^{fx/fx}$ R26R^{EYFP} chimeras, the CD8SP HSA^{hi} thymocytes that were deficient in IKK (CD45.1^{-}) did seem at a competitive disadvantage. However, as predicted from the phenotype of the intact IKKΔT^{CD4} mice, it was the HSA^{lo} SP thymocyte populations that competed least well in the absence of IKK (**Figure 3.6**). In the periphery of the CD4^{Cre} *Ikk1*$^{fx/fx}$ *Ikk2*$^{fx/fx}$ R26R^{EYFP} chimeras, scarcely any CD45.1^{-} cells could be detected, again consistent with the phenotype of the intact IKKΔT^{CD4} animal (**Figure 3.6**; **Figure 3.5**). From the use of competitive chimeras, we can conclude that the phenotype of CD4^{Cre} *Ikk2*$^{fx/fx}$ R26R^{EYFP} mice and IKKΔT^{CD4} mice is indeed T cell intrinsic.

3.2.6 Evidence that IKK signalling acts quantitatively upon T cell development

Quantitative differences in NF-κB activity may be important at different stages in T cell development and function. To investigate this, we generated mice with different combinations of *Ikk1* and *Ikk2* allele deletion mediated by huCD2^{iCre}. We analysed thymic development and peripheral population of the T cell compartment in the different strains. Interestingly, we found evidence of a clear gene dose dependent effect of IKK1 and IKK2 signalling on T cell homeostasis. In the periphery, enumeration of total and naïve T cell numbers revealed a stepwise reduction decreasing in the order: wild-type, *Ikk1*$^{fx/fx}$ *Ikk2*$^{wt/wt}$, *Ikk1*$^{fx/fx}$ *Ikk2*$^{wt/fx}$, *Ikk1*$^{wt/wt}$ *Ikk2*$^{fx/fx}$, *Ikk1*$^{wt/fx}$ *Ikk2*$^{fx/fx}$ to *Ikk1*$^{fx/fx}$ *Ikk2*$^{fx/fx}$ (**Figure 3.7A**). Total numbers of CD4^{+} memory and T_{reg} cells were dramatically reduced in *Ikk1*$^{wt/wt}$ *Ikk2*$^{fx/fx}$ mice, but surprisingly began to increase again in *Ikk1*$^{wt/fx}$ *Ikk2*$^{fx/fx}$ mice. Total numbers of CD8^{+} memory cells were reduced in *Ikk1*$^{wt/fx}$ *Ikk2*$^{fx/fx}$ mice, but increased in *Ikk1*$^{fx/fx}$ *Ikk2*$^{fx/fx}$ mice (**Figure 3.7A**). Examining YFP expression revealed that the increase in memory and T_{reg} populations was due to the presence of YFP- “deletional escapants” (**Figure 3.7A**).

Our previous results have revealed a decreased expression of the IL-7R on the naïve T cells of mice lacking IKK proteins. Compared with the IKK1 deficient naïve cells, the IKK2 deficient naïve cells had less IL-7R expression (**Figure 3.2C**). Furthermore, the CD4 naïve cells from the IKKΔT^{CD4} mice were found to have scarcely any IL-7R expression (**Figure 3.5E**). For this reason we chose to examine IL-7R expression among cells with different extents and combinations of *Ikk1* and *Ikk2* deletion. We observed a stepwise reduction in IL-7R expression in a manner similar to that described for total and naïve T cell numbers (**Figure 3.7B**). As our previous results indicated, the CD8^{+} naïve T cells of *Ikk1*$^{fx/fx}$ *Ikk2*$^{fx/fx}$ mice showed surprisingly high IL-7R expression (**Figure 3.5E**; **Figure 3.7B**). However, this is likely due to the presence of deletion escapants amongst CD8 naïve cells that would have more normal IL-7R expression and therefore survive more efficiently than IKK deficient cells.

In conclusion there is clear evidence of a quantitative impact of IKK signalling on T cell development. In particular, naïve T cell homeostasis and expression of the IL-7R on naïve T cells appeared to closely correlate with the extent of IKK signalling.

3.2.7 Normal development, but reduced naïve T cells in the absence of RelA and NF-κB1

We next wished to examine the role of different Rel family members in development and maintenance of T cells. RelA deficiency is embryonically lethal (Beg et al., 1995). Therefore, we examined a T cell specific deletion of RelA by using the $CD4^{Cre}$ *Rela*$^{fx/fx}$ $R26R^{EYFP}$ strain. In addition, we examined *Nfkb1*$^{-/-}$ mice that lack p105 and p50 (Sha et al., 1995). To examine the effect of a compound deficiency of NF-κB1 and RelA, we also examined $CD4^{Cre}$ *Rela*$^{fx/fx}$ *Nfkb1*$^{-/-}$ $R26R^{EYFP}$ mice. Both early and late stages of thymic development in $CD4^{Cre}$ *Rela*$^{fx/fx}$ *Nfkb1*$^{-/-}$ $R26R^{EYFP}$ mice appeared normal (**Figure 3.8**). Previous studies reveal that *Nfkb1*$^{-/-}$ mice contain a large population of CD8SP thymocytes with a memory like phenotype (Gugasyan et al., 2012). These memory like CD8 cells have an $HSA^{lo}CD44^{hi}CD122^{hi}$ phenotype. Interestingly, in the RelA NF-κB1 double deficient strain this population was absent, suggesting that these cells are RelA dependent (**Figure 3.8A, bottom row**).

Analysing RelA deficient mice revealed a largely normal peripheral T cell compartment, except for a small, but significant reduction in T_{reg} cells (**Figure 3.9A & B**). NF-κB1 deficient mice also had a trend towards reduced T_{reg} cells (**Figure 3.9A & B**). As expected from the thymic phenotype, and as previously described, the *Nfkb1*$^{-/-}$ mice had a large population of CD8 memory cells in the periphery (**Figure 3.9B**). In contrast, $CD4^{Cre}$ *Rela*$^{fx/fx}$ *Nfkb1*$^{-/-}$ $R26R^{EYFP}$ mice had a significantly reduced peripheral T cell compartment, which included a reduced $CD8^{+}$ memory population (**Figure 3.9B**). The naïve cells of the $CD4^{Cre}$ *Rela*$^{fx/fx}$ $R26R^{EYFP}$ mice and the *Nfkb1*$^{-/-}$ mice both had slightly reduced expression of the IL-7R. However, naïve T cells from $CD4^{Cre}$ *Rela*$^{fx/fx}$ *Nfkb1*$^{-/-}$ $R26R^{EYFP}$ mice had a more substantial reduction in IL-7R expression (**Figure 3.9C**).

3.3 Discussion

We began this chapter by comparing the roles IKK1 and IKK2 play in T cell homeostasis. IKK1 and IKK2 are structurally very similar, but have been shown to have distinct functions *in vivo*. This is due to IKK1 having numerous functions independent of kinase activity and NF-κB activation (Oeckinghaus et al., 2011). Together with NEMO, both IKK1 and IKK2 are components of the IKK complex. IKK2 is specific to the canonical NF-κB signalling pathway. However, T cells have been found to compensate for its absence, at least to a certain extent, via formation of IKK1 homodimers (Schmidt-Supprian et al., 2003). In contrast, activation of the NF-κB non-canonical pathway relies on the formation of an IKK1 homodimer and involves neither IKK2 nor NEMO. Thus IKK1 is absolutely required to activate non-canonical NF-κB signalling, and in its absence this pathway is terminated. We hypothesised that IKK1 and IKK2 may have different functions in T cell development and homeostasis and sought to test this by way of mice with a conditional deletion of either protein.

In the past, the use of $IKK1^{-/-}$ bone marrow chimeras had led to the belief that IKK1, although necessary for the normal development of B cells, was dispensable for maturation and homeostasis of the T cell compartment (Kaisho et al., 2001; Senftleben et al., 2001a). Through the use of $huCD2^{iCre}$ *Ikk1*$^{fx/fx}$ $R26R^{EYFP}$ mice, we have shown that thymocyte development was normal in the absence of IKK1, except for the production of T_{reg} cells, which appeared significantly reduced (**Figure 3.1**). Furthermore, we have shown a reduction in $CD4^+$ memory and T_{reg} cells in the periphery of $huCD2^{iCre}$ *Ikk1*$^{fx/fx}$ $R26R^{EYFP}$ mice (**Figure 3.2**, **Table 3.1**). A similar phenotype was recently described in mice with a $CD4^{Cre}$ mediated deletion of IKK1. These mice were shown to have reduced T_{reg} cells and poor expansion of peripheral $CD4^+$ effector cells (Chen et al., 2015).

A lymphoid specific deficiency of IKK2 has previously been described to have no effect on thymocyte development (Schmidt-Supprian et al., 2003; Silva et al., 2014). Here, however, we show that thymic T_{reg} cells are reduced in IKK2

deficient mice and more dramatically so than in IKK1 deficient animals (**Figure 3.1**). Memory and regulatory populations in the periphery have been reported to be reduced in the absence of IKK2 (Schmidt-Supprian et al., 2003). Yet while some studies have seen reduced naïve cells in IKK2 deficient mice (Silva et al., 2014), others have described a normal naïve cell compartment (Schmidt-Supprian et al., 2003). We found the memory and regulatory compartments to be the most affected by the loss of IKK2. However, we also noted a loss of naïve cells - the reduction being greater among the CD8 than the CD4 lineage, as previously reported (Silva et al., 2014).

In previous work, Ren et al., attempted to directly compare the roles of IKK1 and IKK2 in early T cell development. They stated that IKK2, but not IKK1, dependent activation of NF-κB signalling plays a role in the negative selection of DP thymocytes. Ultimately then, they concluded that IKK1 and IKK2 may play opposite roles in thymocyte development (Ren et al., 2002). Here we show that the IKK1 and IKK2 deficient mice present with similar, although not identical, T cell phenotypes (**Figure 3.1**; **Figure 3.2**, **Table 3.1**). This seems to suggest overlapping roles for IKK1 and IKK2 in the later, if not the earlier, stages of T cell development. Past studies suggested that IKK1 and IKK2 are functionally redundant with one another, but also emphasised the more potent kinase activity of the IKK2 subunit (Yamamoto et al., 2000). Our data suggest that IKK1 may not be entirely redundant for peripheral T cell function.

In an attempt to uncover the most important NF-κB subunits in T cells, we investigated mice with a conditional deletion of RelA, a complete deficiency of NF-κB1, and a T cell specific combined deficiency of both RelA and NF-κB1. Thymocyte development appeared normal in these mice, with the exception of the NF-κB1 single knockout animals which, as previously documented, had a large increase in memory like CD8SP cells (Gugasyan et al., 2012). Interestingly, in the periphery of all three strains there was a reduction in $CD4^+$ T_{reg} cells (**Figure 3.9A & B**). The most important NF-κB protein for the production of T_{reg} cells is believed to be c-Rel, followed secondly by RelA (Isomura et al., 2009). Indeed, our results serve to confirm the importance of RelA for T_{reg} production.

Together our results indicate that T_{reg} cells have a great dependency on NF-κB signalling. Loss of IKK1, IKK2, RelA, or NF-κB1 alone all led to a reduction in T_{reg} cells (**Figure 3.1**; **Figure 3.2A & B**; **Figure 3.7A**; **Figure 3.9A & B**). $CD4^+$ memory cells also seem to be particularly NF-κB dependent, since loss of either IKK1 or IKK2 alone led to a significant reduction of this population in the periphery (**Figure 3.2A & B**; **Figure 3.7A**). It is generally accepted that the production of memory and regulatory cells is dependent on antigen induced signalling. IKK2 deficient mice and mice doubly deficient for p50 and c-Rel have previously been described as lacking memory and regulatory compartments (Schmidt-Supprian et al., 2003; Zheng et al., 2003). In such situations it has been proposed that the reduction in these cell types may be due to lack of TCR mediated activation of the canonical NF-κB pathway (Schmidt-Supprian et al., 2003; Zheng et al., 2003). However, loss of IKK1 alone can dramatically reduce $CD4^+$ regulatory and memory cell numbers (Chen et al., 2015). Even in the absence of IKK1, IKK2 and NEMO are thought to cause full TCR induced canonical NF-κB activation (Kaisho et al., 2001; Takeda et al., 1999). Evidence suggests that signalling via receptors of the TNF superfamily activates the non-canonical NF-κB pathway and results in the production and expansion of T_{reg} cells (Chen et al., 2013; Coquet et al., 2013; Mahmud et al., 2014). In the absence of IKK1 the non-canonical NF-κB pathway is blocked. Hence, activation of both canonical and non-canonical NF-κB pathways seems to be required for the production of T_{reg} and $CD4^+$ memory cells.

A further aim of this chapter was to assess the effect of a complete block in NF-κB signalling on T cell development and homeostasis. Mice with a T cell specific, conditional deletion of NEMO have been previously described (Schmidt-Supprian et al., 2003). However, although T cells from these mice would have had a complete block in the canonical pathway of NF-κB activation (Rudolph et al., 2000), the non-canonical pathway would have remained intact. Here we used mice with a T cell specific deficiency of both IKK1 and IKK2 to determine the effect of a complete block in NF-κB signalling. As predicted, the IKK deficient mice closely phenocopied the NEMO deficient animal. Both strains of mice exhibited a clear block in the later stages of thymocyte development and had very few peripheral T cells (**Figure 3.4**; **Figure 3.5**; Schmidt-Supprian

et al., 2003). Through the use of competitive chimeras we have shown that the defects in the IKK deficient mice are indeed T cell intrinsic (**Figure 3.6**).

The role of NF-κB signalling during thymic selection has proven controversial. Virtually all studies into the effects of cell intrinsic NF-κB signalling on thymocyte selection have used TCR transgenic mice expressing a super-inhibitory form of the IκB proteins. In the T cells of these mice, both TCR alpha and beta chains are pre-arranged and therefore expressed early in development - hence leading to a rather non-physiological situation. It is possible that these studies have been over complicated by the inclusion of the transgenic TCRs. For example, positive selection is often measured by CD69 expression (Hettmann and Leiden, 2000; Mora et al., 2001). However, it has been suggested that the observed reduction in CD69 may not be a direct effect of reduced NF-κB, but rather due to defects in TCR signalling that, with a more physiologically relevant model, may not have been present (Mora et al., 2001).

We therefore wished to assess the effect of blocking NF-κB signalling early in thymocyte development through the use of early deleting Cre recombinases and in the absence of any transgenic TCRs. DP thymocytes were present in normal numbers in IKK deficient mice (**Figure 3.3A & D**). Early CD4SP populations (HSA^{hi}) were also found to be unchanged between the IKK deficient mice and their Cre- littermates (**Figure 3.4B & C**). This suggests that NF-κB signalling is dispensable during positive selection. TCR transgenic mice, with a $huCD2^{iCre}$ mediated deficiency of IKK2, have been previously described (Silva et al., 2014). The transgenic T cells in these mice also underwent normal positive selection, providing further evidence that IKK activity is redundant at this stage of T cell development.

The normal numbers of DN3 and DN4 thymocytes in the $IKK\Delta T^{CD2}$ mice hints that NF-κB is not required for beta-selection (**Figure 3.3A & C**). We compared our $huCD2^{iCre}$ mice to mice with a $CD4^{Cre}$ mediated deficiency of NF-κB signalling. Doing so revealed no phenotypic differences between the strains (**Figure 3.3D**; data not shown). Hence it seems of little consequence whether *Ikk1* and *Ikk2* are deleted during the DN stage of thymocyte development (by

huCD2^{iCre}) or during the DP stage (by CD4^{Cre}). However, despite our results, we cannot state with certainty that there is no function for NF-κB during thymic selection stages. To do so would require firstly, proof that NF-κB signals have actually been terminated by the late DN stages of development in the huCD2^{iCre} mice and secondly, evidence that normal selection has indeed occurred.

Previous studies have shown that IKK2 deficient naïve T cells have reduced expression of the IL-7R (Miller et al., 2014; Silva et al., 2014). This has been found to correlate with a reduction in *Il7r* mRNA levels and to be cell intrinsic (Silva et al., 2014). When we examined the IKK1 deficient mice we found reduced IL-7R expression on the CD8 naïve cells (**Figure 3.2C**). The IKK1/2 double deficient mice had scarcely any peripheral cells present, however the few remaining CD4 naïve cells showed extremely low levels of the IL-7R (**Figure 3.5E**). In physiological situations, the amount of circulating IL-7 cytokine controls the level of IL-7R expression. In the presence of increased IL-7, IL-7Rα is down-regulated (Park et al., 2004). However, use of mixed chimeras showed that the reduced IL-7R levels on the IKK1/2 double deficient mice were not a consequence of increased IL-7 signalling in the lymphopenic environment (data not shown).

Evidence suggests that IKK mediated NF-κB signalling is necessary for the maturation of new T cells by allowing upregulation of the IL-7R (Miller et al., 2014; Silva et al., 2014). We therefore looked at IL-7R expression levels on mice with different degrees of *Ikk1* and *Ikk2* deletion. As expected, we found that IL-7R levels decreased in mice bred with a progressive loss of *Ikk1* and *Ikk2* alleles (**Figure 3.7B**). Moreover, we found that loss of naïve cells correlated with the loss of IL-7R (**Figure 3.7**). Surprisingly, IL-7R expression remained fairly high on CD8 naïve IKK1/2 double deficient cells (**Figure 3.5E**; **Figure 3.7B**). It is possible that, due to selection pressures, the few remaining cells within the CD8^{+} naïve population were those expressing the greatest amount of IL-7R or were cells that failed to fully delete all *Ikk1* and *Ikk2* alleles.

IL-7R expression is of particular importance for maintenance of the naïve T cell population (Jameson, 2005). Thus we believe that loss of IL-7R expression

could provide one, if not the sole explanation for the loss of naïve cells. Interestingly, mice deficient in either RelA or NF-κB1 had only a slight defect in IL-7R expression and normal numbers of naïve cells (**Figure 3.9**). The combined absence of both RelA and NF-κB1 did result in a more striking phenotype (**Figure 3.9**). However, compared to the IKK1/2 double deficient mice, the RelA NF-κB1 double deficient animals were much less affected (**Figure 3.4**; **Figure 3.5**; **Figure 3.8**; **Figure 3.9**). It seemed that the loss of both RelA and NF-κB1 resulted in a reduction in NF-κB activity similar to that of the IKK2 deficient mice – i.e. partial, but not complete (**Figure 3.1**; **Figure 3.2**; **Figure 3.8**; **Figure 3.9**; **Table 3.1**). This serves to confirm the redundancy that exists between the different members of the Rel protein family.

IKK2 has been noted to have between 20 and 50 fold greater kinase activity for IκB than does IKK1 (Yamamoto et al., 2000). Therefore, we anticipated the loss of *Ikk2* alleles to be of greater significance than the loss of *Ikk1* alleles. Our data supports this – notably mice with no *Ikk1* alleles and only one *Ikk2* allele ($Ikk1^{fx/fx}$ $Ikk2^{wt/fx}$) had more T cells and greater IL-7R expression than mice with both *Ikk1* alleles, but no *Ikk2* alleles ($Ikk1^{wt/wt}$ $Ikk2^{fx/fx}$) (**Figure 3.7**). IL-7R expression appeared to correlate with NF-κB signalling strength. The IKK complex can consist of IKK1 or IKK2 homodimers as well as the common IKK1/2 heterodimer. The subtle changes in IKK1 and IKK2 expression that occur in wt/fx mice are likely to affect the composition and therefore function of the IKK complex. Indeed the unique phenotypes of the mice with different extents and combinations of *Ikk* gene deletion provide strong evidence for this (**Figure 3.7**).

Other studies have also shown gene dose dependent effects of NF-κB pathway components on T cell homeostasis. In mice overexpressing IκBα, the reduction in peripheral T cell number was found to be transgene dose dependent – animals expressing 80 copies of IκBα per haploid chromosome complement showing more severe phenotypes than those expressing 25 copies (Esslinger et al., 1997). Furthermore, whilst hemizygous animals had 3 times fewer $CD4^+$ cells and 10 times fewer $CD8^+$ cells than control animals, homozygous animals had virtually no peripheral αβ T cells (Esslinger et al., 1997). Such results argue

in favour of a threshold level requirement for NF-κB signalling. Furthermore, genetic ablation of IKK2 is shown to be better tolerated than replacing IKK2 with a kinase dead mutant (Schmidt-Supprian et al., 2003). This suggests that compensatory signalling can occur by IKK1 homodimers. In conclusion, we believe that there may be clear gene dose dependent effects of IKK1/2 signalling on T cell development.

A certain threshold of cell intrinsic NF-κB signalling seems to be necessary for the maturation and survival of SP thymocytes. When NF-κB signalling is completely blocked in T cells, by a deletion of the gene encoding NEMO, or by deletion of the genes for both IKK1 and IKK2, there is a significant reduction in thymic SP populations (Schmidt-Supprian et al., 2003). The receptor responsible for activating NF-κB in this context is not known. However, it appears to be independent of the TCR since deletion of Bcl-10, required for TCR mediated NF-κB activation, does not prevent maturation of the SP population (Ruland et al., 2001; Schmidt-Supprian et al., 2003).

In conclusion, the results presented in this chapter show that there is a T cell intrinsic requirement for NF-κB signalling for thymic development and peripheral T cell homeostasis. In particular, we have discovered that IKK signalling is required during the later stages of thymocyte maturation. In the absence of both IKK1 and IKK2, there is a block between the HSA^{hi} and HSA^{lo} stages of SP development, similar to the block seen in NEMO deficient animals (Schmidt-Supprian et al., 2003). The mechanism of this block is unclear, as is the identity of the receptor required to activate NF-κB. In **Chapter 4** we will therefore attempt to address these unknowns.

Figure 3.1 Mice with a conditional deletion of either *Ikk1* or *Ikk2* have a reduction in thymic T_{reg} cells

The thymii of 7-25 week old: huCD2^{iCre} *Ikk1*$^{fx/fx}$ R26R^{EYFP} mice, huCD2^{iCre} *Ikk2*$^{fx/fx}$ R26R^{EYFP} mice, and their Cre- (WT) littermates were analysed by FACS. (**A**) From top to bottom, density plots show: CD4 vs CD8 expression by total live thymocytes; CD25 vs CD44 expression by CD4SP thymocytes; HSA vs TCR expression by CD4SP thymocytes; HSA vs TCR expression by CD8SP thymocytes. (**B**) Bar charts display the number of cells within the given thymic populations.

Cre- (WT), (n = 14); huCD2^{iCre} *Ikk1*$^{fx/fx}$, (n = 14); huCD2^{iCre} *Ikk2*$^{fx/fx}$, (n = 9). Data was collected from 6 independent FACS experiments. For all density plots, numbers represent the percentage of cells in the corresponding gate. Bar charts show mean cell number ± SEM. ns = not significant; * = significant at P<0.05; ** = significant at P<0.01; *** = significant at P<0.001.

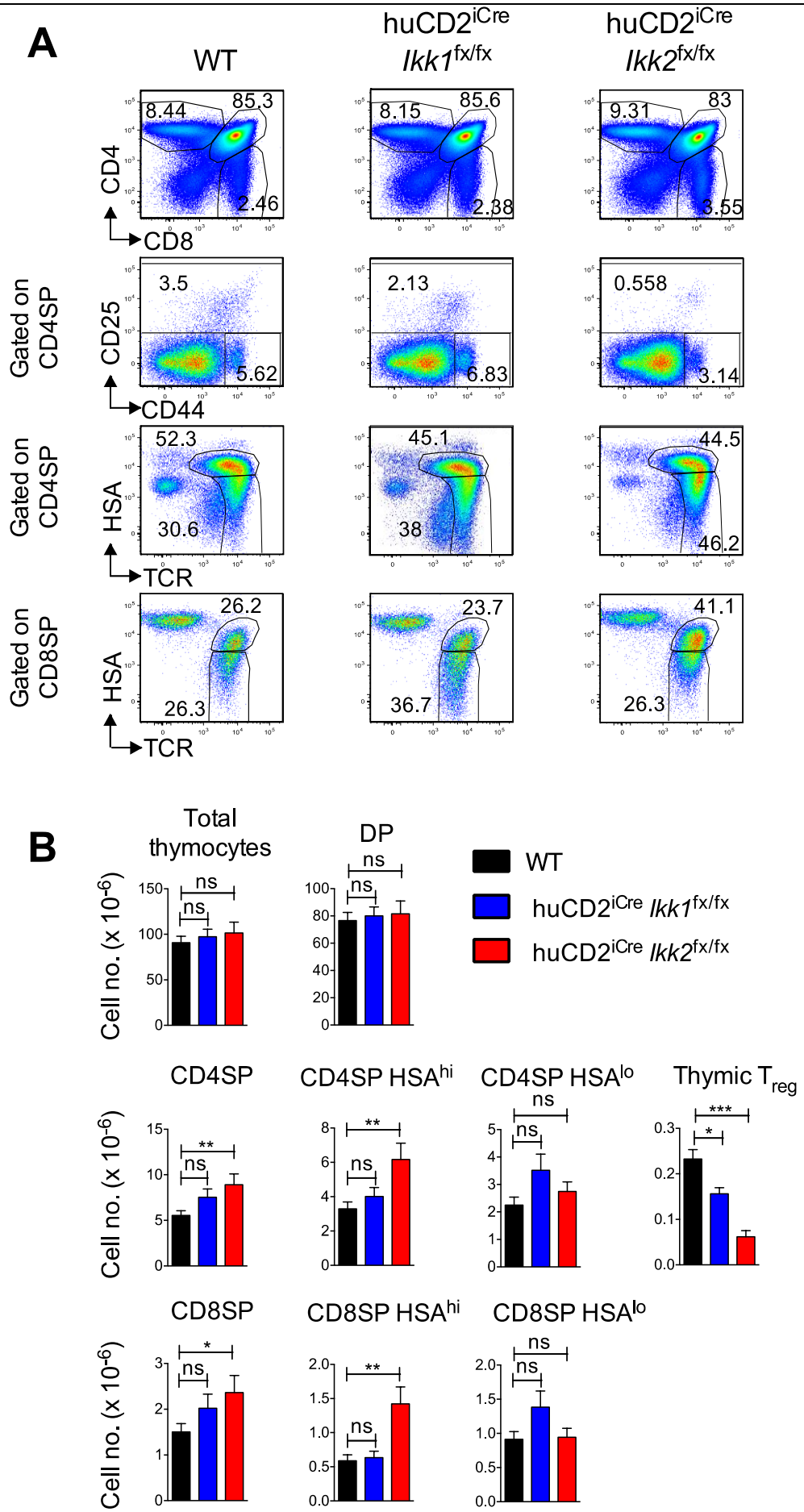

A
WT
huCD2iCre Ikk1fx/fx
huCD2iCre Ikk2fx/fx
CD4
CD8
8.44
85.3
2.46
8.15
85.6
2.38
9.31
83
3.55
Gated on CD4SP
CD25
CD44
3.5
5.62
2.13
6.83
0.558
3.14
Gated on CD4SP
HSA
TCR
52.3
30.6
45.1
38
44.5
46.2
Gated on CD8SP
HSA
TCR
26.2
26.3
23.7
36.7
41.1
26.3
B
Total thymocytes
DP
WT
huCD2iCre Ikk1fx/fx
huCD2iCre Ikk2fx/fx
Cell no. (x 10-6)
ns
CD4SP
CD4SP HSAhi
CD4SP HSAlo
Thymic Treg
CD8SP
CD8SP HSAhi
CD8SP HSAlo
*
**
***

Figure 3.2 Mice with a conditional deletion of either *Ikk1* or *Ikk2* have a reduction in $CD4^+$ memory and T_{reg} cells in the periphery

The LNs and spleen were taken from 7-25 week old: $huCD2^{iCre}$ $Ikk1^{fx/fx}$ $R26R^{EYFP}$ mice, $huCD2^{iCre}$ $Ikk2^{fx/fx}$ $R26R^{EYFP}$ mice, and their Cre- (WT) littermates and were analysed by FACS. (**A**) Density plots show LN profiles of the indicated mouse strains. From top to bottom, plots show: CD4 vs CD8 expression by TCR^+ cells; CD25 vs CD44 expression by TCR^+CD4^+ cells; TCR vs CD44 expression by TCR^+CD8^+ cells. (**B**) For the different T cell populations, cell counts within the LN and spleen were combined and displayed via bar charts. (**C**) Within the LNs, IL-7R expression on naïve $CD4^+$ or $CD8^+$ T cells is shown for the indicated mice. Bar charts show the mean fluorescence intensity (MFI) of IL-7R staining as a percentage of that seen on "WT" cells (Cre- cells prepared, stained, and analysed concomitantly).

Cre- (WT), (n = 19); $huCD2^{iCre}$ $Ikk1^{fx/fx}$, (n = 19); $huCD2^{iCre}$ $Ikk2^{fx/fx}$, (n = 9). Data was collected from 7 independent FACS experiments. For all density plots, numbers represent the percentage of cells in the corresponding gate. Bar charts show mean cell number ± SEM. ns = not significant; * = significant at $P<0.05$; ** = significant at $P<0.01$; *** = significant at $P<0.001$.

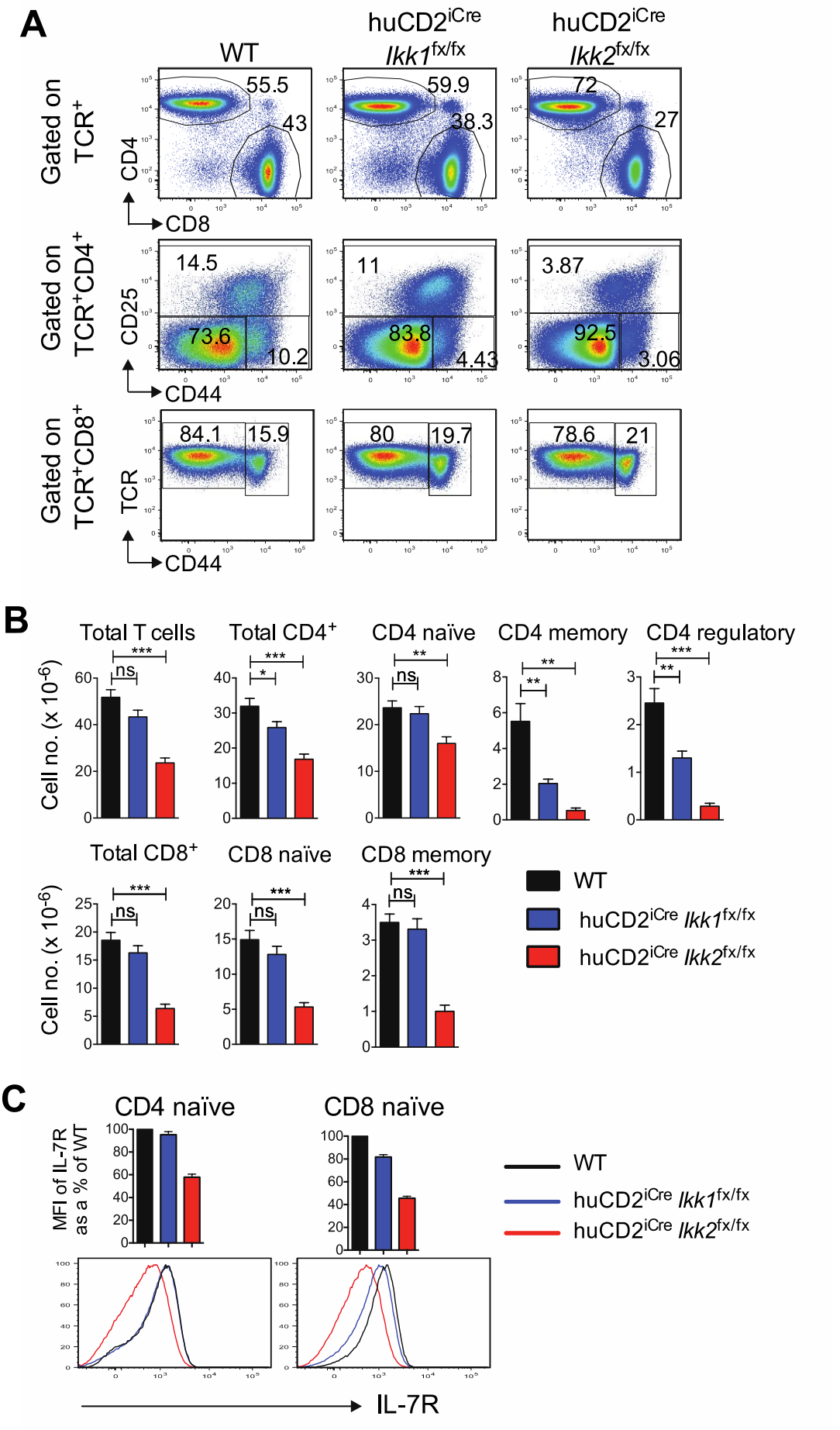

A
WT
huCD2iCre Ikk1fx/fx
huCD2iCre Ikk2fx/fx
Gated on TCR+
CD4
CD8
55.5
43
59.9
38.3
72
27
Gated on TCR+CD4+
CD25
CD44
14.5
73.6
10.2
11
83.8
4.43
3.87
92.5
3.06
Gated on TCR+CD8+
TCR
CD44
84.1
15.9
80
19.7
78.6
21
B
Total T cells
Total CD4+
CD4 naïve
CD4 memory
CD4 regulatory
Total CD8+
CD8 naïve
CD8 memory
Cell no. (x 10-6)
ns
*
**
***
WT
huCD2iCre Ikk1fx/fx
huCD2iCre Ikk2fx/fx
C
CD4 naïve
CD8 naïve
MFI of IL-7R as a % of WT
IL-7R
WT
huCD2iCre Ikk1fx/fx
huCD2iCre Ikk2fx/fx

Figure 3.3 Thymocyte development through the late DN stages and the DP stages is unaffected by loss of both *Ikk1* and *Ikk2*

Early thymocyte development was assessed in 12-26 week old: huCD2^{iCre} *Ikk1*$^{fx/fx}$ *Ikk2*$^{fx/fx}$ R26R^{EYFP} (IKKΔT^{CD2}) mice, CD4^{Cre} *Ikk1*$^{fx/fx}$ *Ikk2*$^{fx/fx}$ R26R^{EYFP} (IKKΔT^{CD4}) mice, and Cre- (WT) littermates. (**A**) Top row shows CD44 vs CD25 expression by TCR^{-}CD5^{-} thymocytes within the live population of CD4^{-}CD8^{-} thymocytes. DN1 = CD44^{+}CD25^{-}, DN2 = CD44^{+}CD25^{+}, DN3 = CD44^{-}CD25^{+}, DN4 = CD44^{-}CD25^{-}. Bottom row shows CD5 vs TCR expression by CD4^{+}CD8^{+} live thymocytes. DP1 = TCR^{lo}CD5^{lo}, DP2 = TCR^{int}CD5^{hi}, DP3 = TCR^{hi}CD5^{int}. (**B**) Histograms show YFP expression within the DN populations of IKKΔT^{CD2} mice vs Cre- littermate controls. Bar charts show the percentage of YFP positive cells within the DN1-4 populations of IKKΔT^{CD2} mice. (**C**) Bar charts show DN cell numbers in IKKΔT^{CD2} (Cre+) mice vs Cre- littermate controls. (**D**) Bar charts show DP cell numbers in IKKΔT^{CD2} mice vs Cre- littermate controls and IKKΔT^{CD4} mice vs Cre- littermate controls.

IKKΔT^{CD2} mice, (n = 16); Cre- (WT) littermates of IKKΔT^{CD2} mice, (n = 15); IKKΔT^{CD4} mice, (n = 15); Cre- (WT) littermates of IKKΔT^{CD4} mice, (n = 11). Numbers on the density plots represent percentages within each gated population. Bar charts show mean ± SEM. ns = not significant; * = significant at P<0.05; ** = significant at P<0.01.

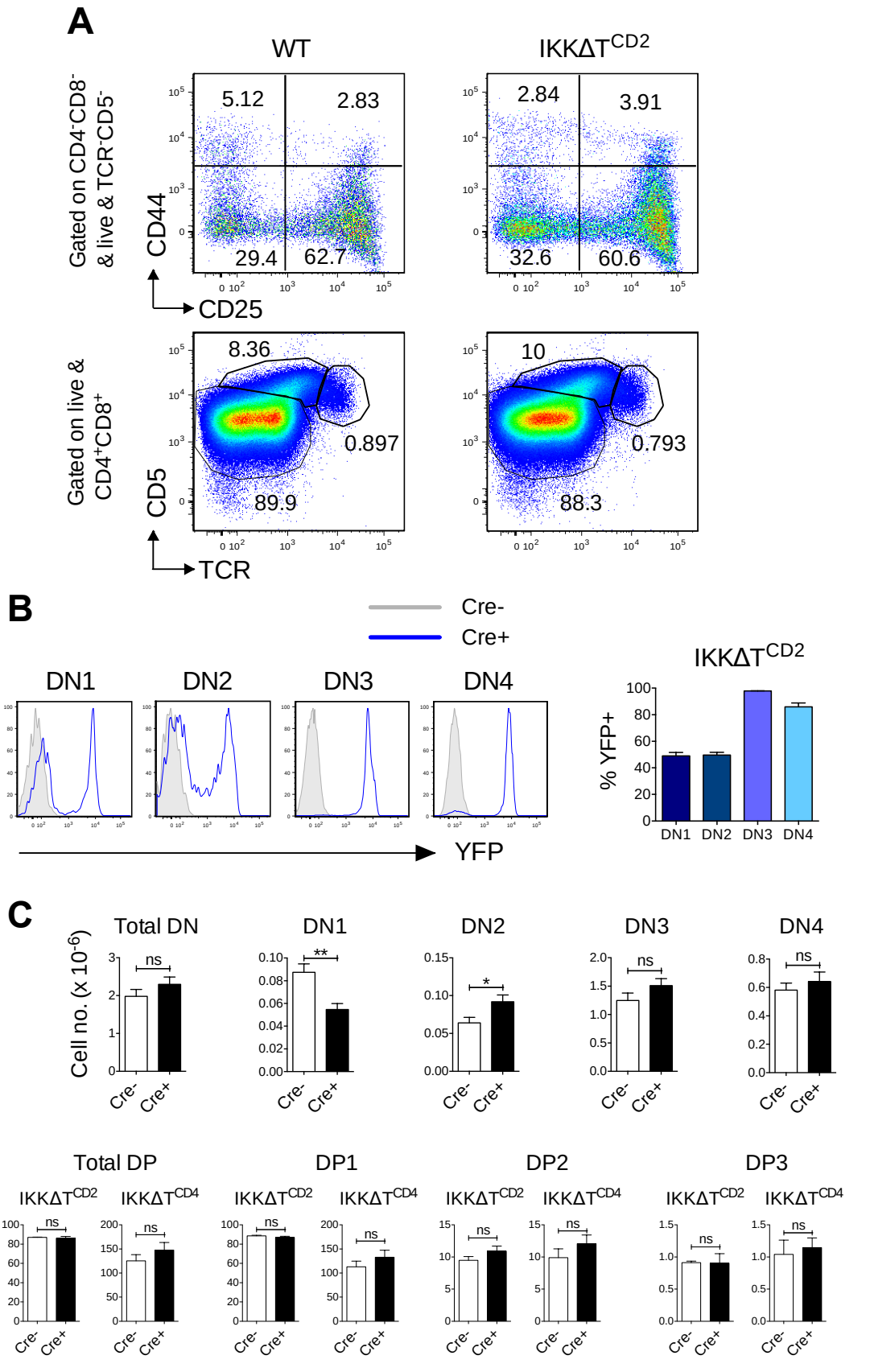

A
WT
IKKΔT^CD2
Gated on CD4-CD8- & live & TCR-CD5-
CD44
CD25
5.12
2.83
29.4
62.7
2.84
3.91
32.6
60.6
Gated on live & CD4+CD8+
CD5
TCR
8.36
0.897
89.9
10
0.793
88.3
B
Cre-
Cre+
DN1
DN2
DN3
DN4
YFP
IKKΔT^CD2
% YFP+
C
Total DN
DN1
DN2
DN3
DN4
Cell no. (x 10-6)
ns
**
*
Cre-
Cre+
D
Total DP
DP1
DP2
DP3
IKKΔT^CD2
IKKΔT^CD4
Cell no. (x 10-6)
ns

Figure 3.4 Conditional deletion of both *Ikk1* and *Ikk2* early in T cell development leads to a severe block in the later stages of thymocyte maturation

The thymii of 10-14 week old $CD4^{Cre}$ $Ikk1^{fx/fx}$ $Ikk2^{fx/fx}$ $R26R^{EYFP}$ ($IKK\Delta T^{CD4}$) mice and Cre- (WT) littermates were analysed by FACS. (**A**) Moving from top to bottom, density plots show: CD4 vs CD8 expression by total live thymocytes; CD5 vs TCR expression by total live thymocytes; CD4 vs CD8 expression by $TCR^{hi}CD5^{hi}$ thymocytes. (**B**) Top row shows HSA vs TCR expression by CD4SP thymocytes. Bottom row shows HSA vs TCR expression by CD8SP thymocytes. (**C**) Total numbers of live thymocytes and numbers of HSA^{hi} and HSA^{lo} cells within the mature CD4SP and CD8SP populations are shown for $IKK\Delta T^{CD4}$ (Cre+) mice and Cre- littermate controls. For the Cre+ mice, stacked bars are used to indicate the numbers of YFP+ and YFP- cells. (**D**) The block in thymocyte development in the $IKK\Delta T^{CD4}$ mice is illustrated.

Cre- (WT) mice, (n = 18); Cre+ ($IKK\Delta T^{CD4}$) mice, (n = 24). Numbers on the density plots represent percentages within each gated population. Bar charts show mean cell number ± SEM. ns = not significant; *** = significant at $P<0.001$.

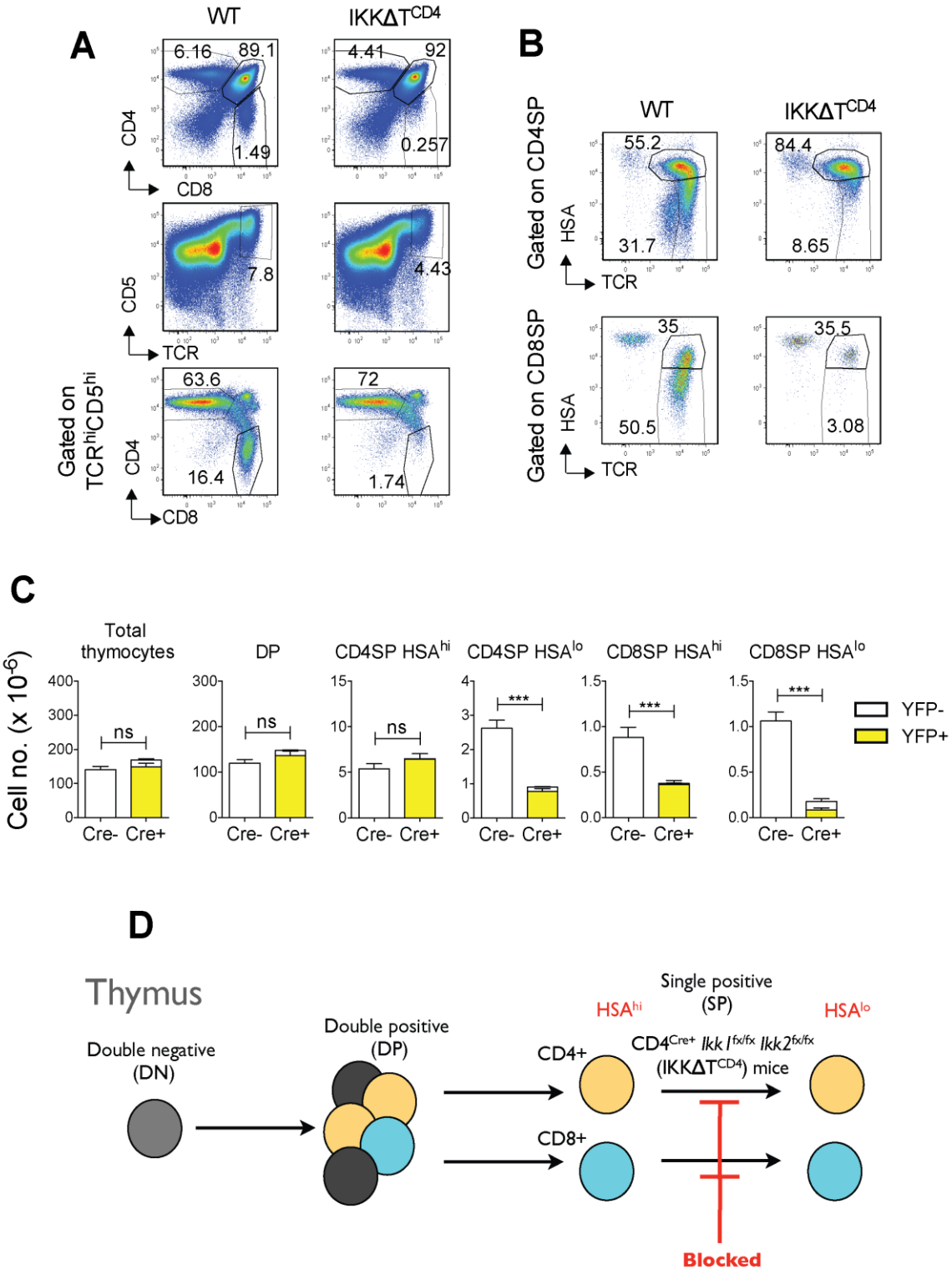

A
WT
IKKΔT^CD4
6.16
89.1
1.49
4.41
92
0.257
CD4
CD8
7.8
4.43
CD5
TCR
63.6
16.4
72
1.74
Gated on TCR^hiCD5^hi
CD4
CD8
B
WT
IKKΔT^CD4
Gated on CD4SP
55.2
31.7
84.4
8.65
HSA
TCR
Gated on CD8SP
35
50.5
35.5
3.08
HSA
TCR
C
Cell no. (x 10^-6)
Total thymocytes
DP
CD4SP HSA^hi
CD4SP HSA^lo
CD8SP HSA^hi
CD8SP HSA^lo
ns
***
Cre- Cre+
YFP-
YFP+
D
Thymus
Double negative (DN)
Double positive (DP)
Single positive (SP)
HSA^hi
HSA^lo
CD4+
CD8+
CD4^Cre+ Ikk1^fx/fx Ikk2^fx/fx (IKKΔT^CD4) mice
Blocked

Figure 3.5 Conditional deletion of both *Ikk1* and *Ikk2* early in T cell development causes a significant reduction in peripheral T cell numbers

The LNs and spleen of 10-14 week old $CD4^{Cre+}$ $Ikk1^{fx/fx}$ $Ikk2^{fx/fx}$ $R26R^{EYFP}$ ($IKK\Delta T^{CD4}$) mice and Cre- (WT) littermates were analysed by FACS. (**A-C** and **E**) The T cell phenotype within the LN is shown. (**A**) Top row shows CD5 vs TCR expression by total live lymphocytes. Bottom row shows CD4 vs CD8 expression by $TCR^{hi}CD5^{hi}$ cells. (**B**) Top row shows CD25 vs CD44 expression by $CD4^+$ cells. Bottom row shows TCR vs CD44 expression by $CD8^+$ cells. (**C**) Histograms are of YFP expression by WT (Cre-, grey fill) and Cre+ (black line) mice. YFP expression within the $CD4^+$ naïve, memory, and regulatory T cell populations and the $CD8^+$ naïve and memory T cell populations is shown. $CD4^{Cre+}$ $Ikk1^{wt/wt}$ $Ikk2^{wt/fx}$ $R26R^{EYFP}$ mice (top) are controls for $IKK\Delta T^{CD4}$ mice (bottom). Numbers indicate the percentage of YFP+ cells within the T cell populations of Cre+ mice. (**D**) Peripheral T cell numbers are shown for $IKK\Delta T^{CD4}$ (Cre+) mice and WT (Cre-) littermates. These represent total T cell numbers in the LNs and spleen combined. For the Cre+ mice, numbers include both YFP+ and YFP- cells. (**E**) Histograms show expression of the IL-7R on naïve T cells within the LNs. For the Cre+ mice, the naïve cell gates included both YFP+ and YFP- subsets.

Cre- (WT) mice, (n = 17); Cre+ ($IKK\Delta T^{CD4}$) mice, (n = 22). Numbers on the density plots represent percentages within each gated population. Bar charts show mean cell number ± SEM. * = significant at $P<0.05$; *** = significant at $P<0.001$.

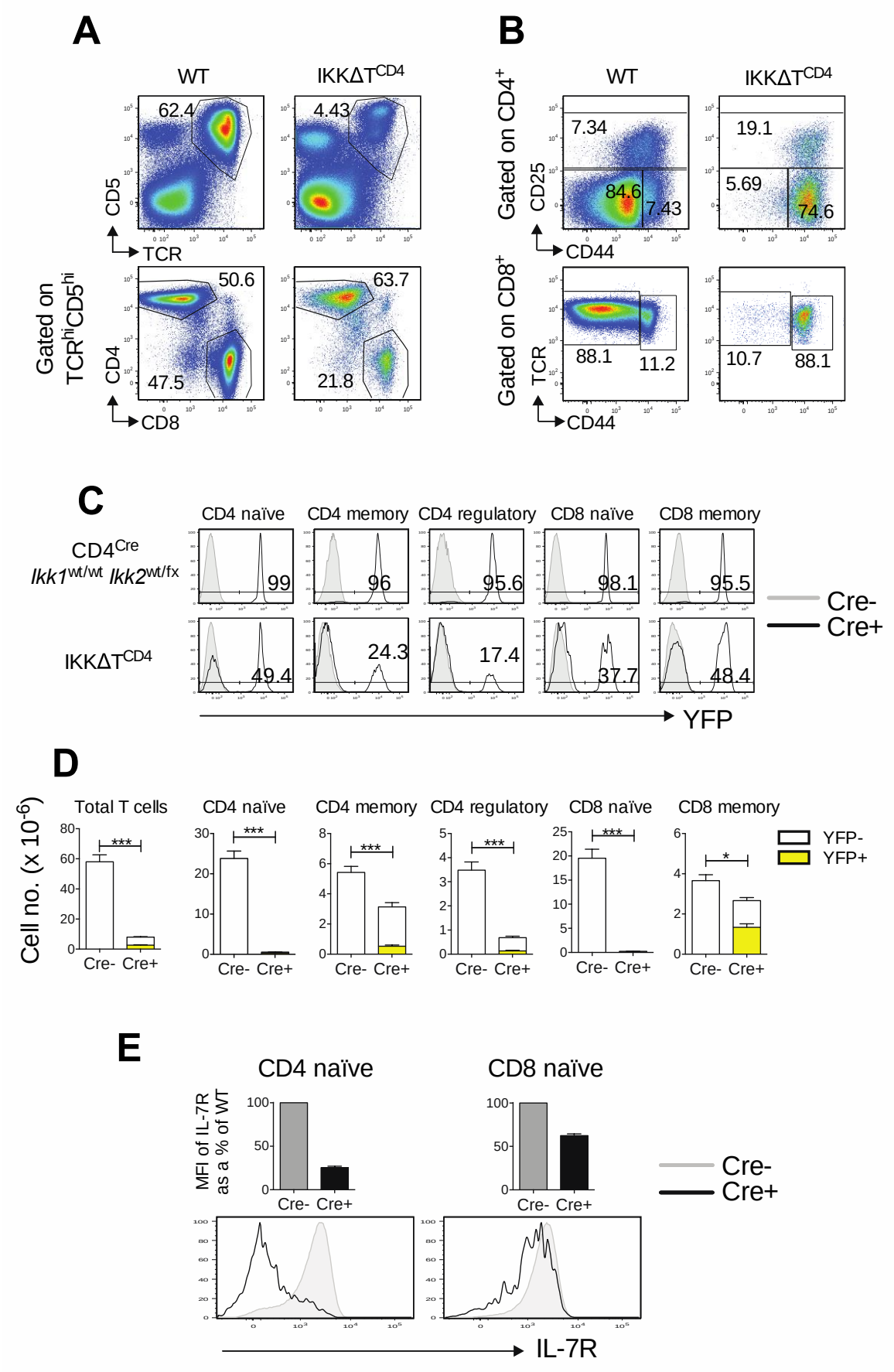

A
WT
IKKΔT^CD4
62.4
4.43
CD5
TCR
Gated on TCR^hiCD5^hi
50.6
63.7
47.5
21.8
CD4
CD8
B
WT
IKKΔT^CD4
Gated on CD4+
7.34
19.1
84.6
7.43
5.69
74.6
CD25
CD44
Gated on CD8+
88.1
11.2
10.7
88.1
TCR
CD44
C
CD4 naïve
CD4 memory
CD4 regulatory
CD8 naïve
CD8 memory
CD4^Cre
Ikk1^wt/wt Ikk2^wt/fx
99
96
95.6
98.1
95.5
IKKΔT^CD4
49.4
24.3
17.4
37.7
48.4
Cre-
Cre+
YFP
D
Cell no. (x 10^-6)
Total T cells
CD4 naïve
CD4 memory
CD4 regulatory
CD8 naïve
CD8 memory
***
*
Cre-
Cre+
YFP-
YFP+
E
CD4 naïve
CD8 naïve
MFI of IL-7R as a % of WT
Cre-
Cre+
IL-7R

Figure 3.6 The defects seen in the CD4^{Cre} *Ikk2*$^{fx/fx}$ R26R^{EYFP} mice and the IKKΔT^{CD4} mice are T cell intrinsic

Mixed haematopoietic chimeras were generated by injecting T cell depleted bone marrow from two different strains of donor mice into the tail vein of irradiated *Rag1*$^{-/-}$ hosts. In each case, bone marrow from congenic CD45.1^{+} C57BL/6 (WT) mice was combined with bone marrow from CD45.1^{-} mice with floxed *Ikk* alleles. Some *Rag1*$^{-/-}$ hosts received a 1:1 ratio of CD45.1^{-} CD4^{Cre} *Ikk2*$^{fx/fx}$ R26R^{EYFP} bone marrow and CD45.1^{+} bone marrow – producing CD4^{Cre+} *Ikk2*$^{fx/fx}$ chimeras. Other hosts received a 1:1 ratio of CD45.1^{-} CD4^{Cre} *Ikk1*$^{fx/fx}$ *Ikk2*$^{fx/fx}$ R26R^{EYFP} (IKKΔT^{CD4}) bone marrow and CD45.1^{+} bone marrow – producing CD4^{Cre} *Ikk1*$^{fx/fx}$ *Ikk2*$^{fx/fx}$ R26R^{EYFP} chimeras. The CD45.1^{-} Cre- littermates of the Cre+ mice also possessed floxed *Ikk* alleles, and their bone marrow was too combined in a 1:1 ratio with CD45.1^{+} bone marrow - producing control (Cre-) chimeras. For each set of chimeras produced, the percentage of CD45.1^{-} (transgenic) cells was compared with the percentage of CD45.1^{+} (WT) cells. The percentage of CD45.1^{-} cells within different T cell subsets in the thymus and periphery is shown. The periphery represents T cells in both the LNs and spleen. Chimeras were analysed 8 weeks after receiving donor bone marrow.

Control chimeras, (n = 4); CD4^{Cre+} *Ikk2*$^{fx/fx}$ chimeras, (n = 5); CD4^{Cre} *Ikk1*$^{fx/fx}$ *Ikk2*$^{fx/fx}$ R26R^{EYFP} chimeras, (n = 3). Line graphs show mean percentage ± SEM.

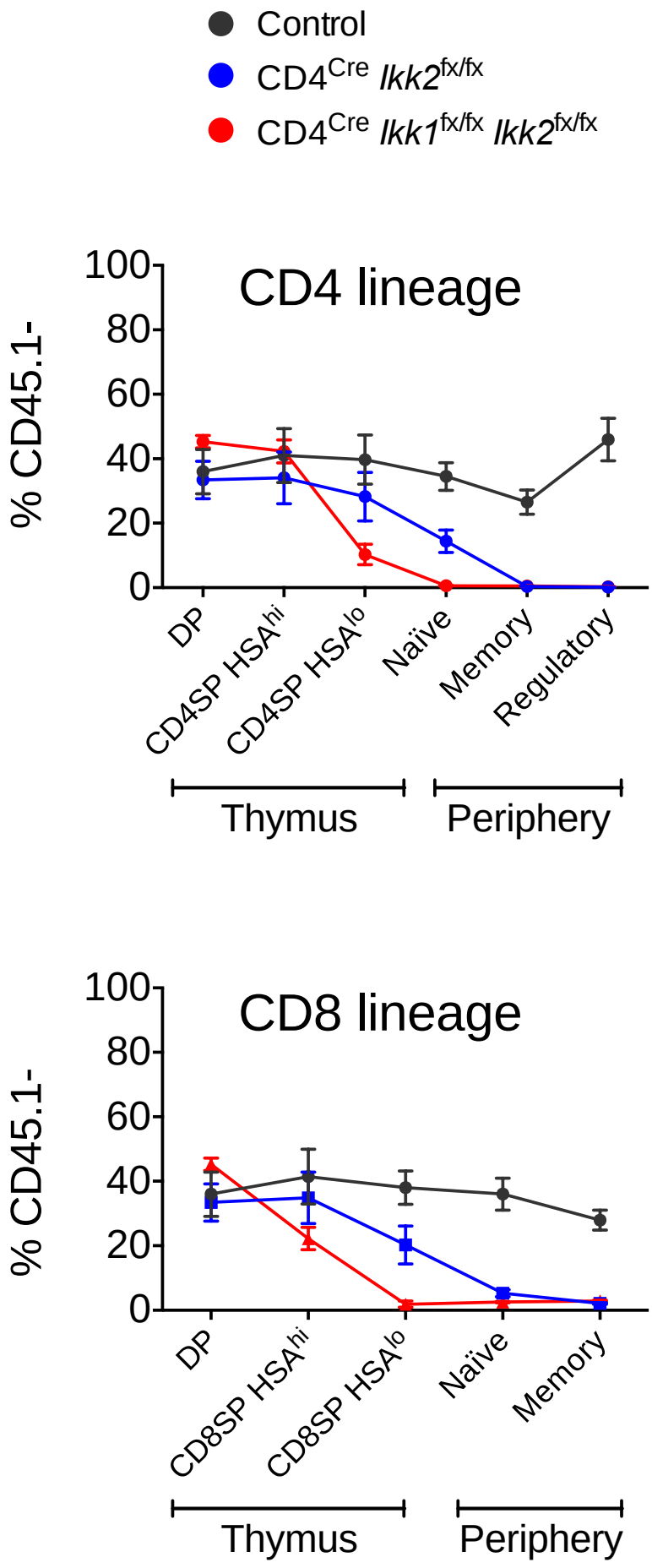

Control
CD4Cre Ikk2fx/fx
CD4Cre Ikk1fx/fx Ikk2fx/fx
CD4 lineage
% CD45.1-
100
80
60
40
20
0
DP
CD4SP HSAhi
CD4SP HSAlo
Naïve
Memory
Regulatory
Thymus
Periphery
CD8 lineage
% CD45.1-
100
80
60
40
20
0
DP
CD8SP HSAhi
CD8SP HSAlo
Naïve
Memory
Thymus
Periphery

Figure 3.7 An apparent gene dose effect of IKK1/2 signalling on T cell development and expression of the IL-7R

7-26 week old huCD2^{iCre} mice homozygous or heterozygous for floxed *Ikk1* and/or *Ikk2* alleles were examined by FACS. A "+" symbol represents the presence of an allele whilst a "-" symbol represents its absence. The first bar of each graph shows mice with a complete set of *Ikk1* and *Ikk2* alleles. These mice are the Cre- littermates of the Cre+ mice, they contain varying numbers of floxed alleles, but no Cre recombinase. The key below the first graph also applies to all other graphs. (**A**) Graphs show T cell numbers in the LNs and spleen of the indicated mice. For the Cre+ mice, stacked bars are used to indicate the numbers of YFP+ and YFP- cells. (**B**) Within the LNs, naïve T cell populations were examined for their expression of the IL-7R. For Cre+ cells, the mean fluorescence intensity (MFI) of IL-7R staining was expressed as a percentage of that seen on "WT" cells (Cre- cells prepared, stained, and analysed concomitantly).

Ikk1 ++ *Ikk2* ++, (n = 42); *Ikk1* -- *Ikk2* ++, (n = 16); *Ikk1* -- *Ikk2* +-, (n = 12); *Ikk1* ++ *Ikk2* --, (n = 9); *Ikk1* +- *Ikk2* --, (n = 16); *Ikk1* -- *Ikk2* --, (n = 13). Bar charts show mean ± SEM.

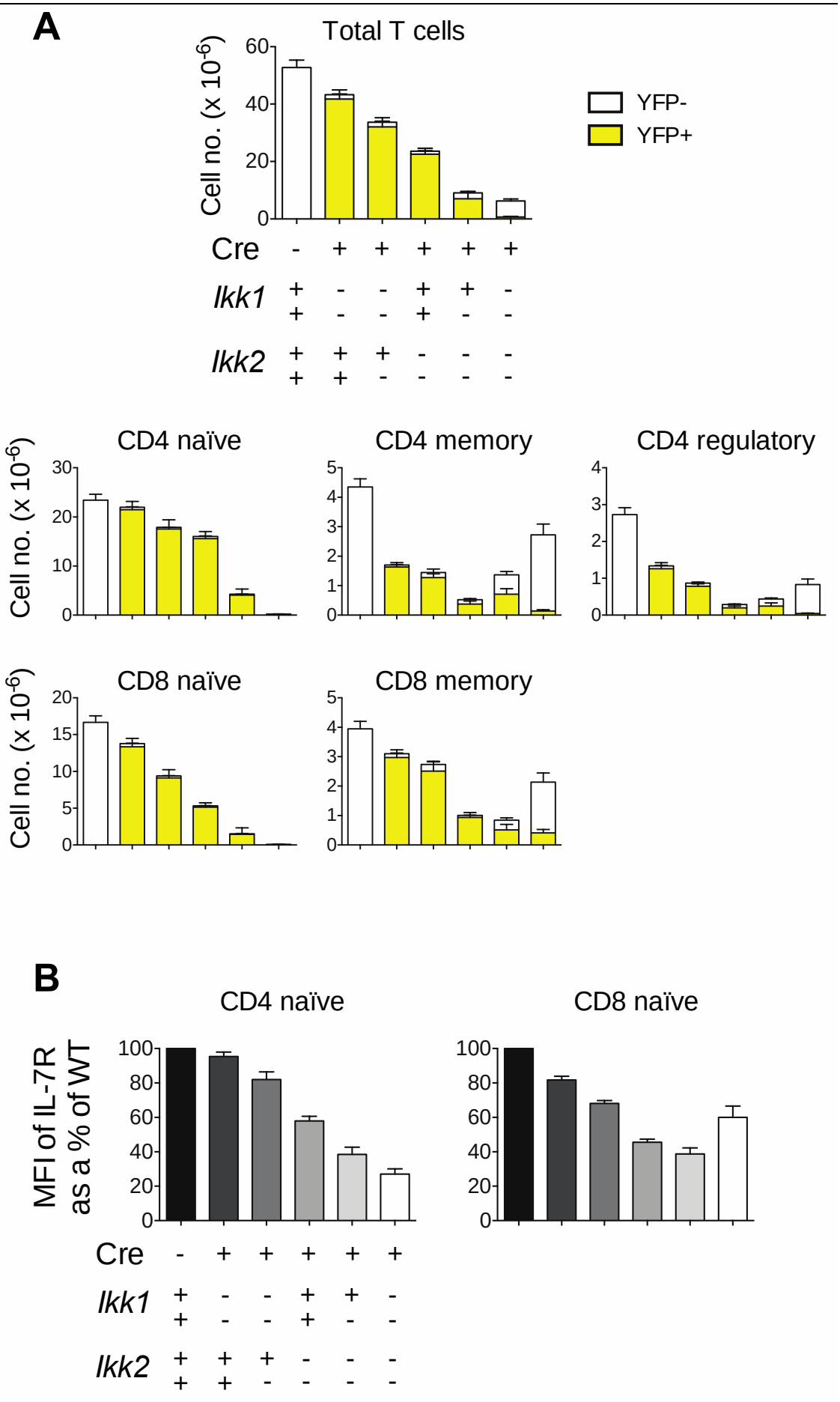

A
Total T cells
Cell no. (x 10-6)
YFP-
YFP+
Cre
Ikk1
Ikk2
CD4 naïve
CD4 memory
CD4 regulatory
CD8 naïve
CD8 memory
B
CD4 naïve
CD8 naïve
MFI of IL-7R as a % of WT
Cre
Ikk1
Ikk2

Figure 3.8 Mice with a deficiency of both NF-κB1 and RelA have normal thymocyte development

Mice with a conditional deficiency of RelA ($CD4^{Cre}$ $Rela^{fx/fx}$ $R26R^{EYFP}$) were crossed with mice in which NF-κB1 had been knocked out ($Nfkb1^{-/-}$) to produce mice deficient in both RelA and NF-κB1 ($CD4^{Cre}$ $Rela^{fx/fx}$ $Nfkb1^{-/-}$ $R26R^{EYFP}$). (**A**) Moving from top to bottom, density plots show: CD4 vs CD8 expression by total live thymocytes; HSA vs TCR expression by CD4SP thymocytes; HSA vs TCR expression by CD8SP thymocytes; CD44 vs CD122 expression by TCR^{hi}CD8SP thymocytes. (**B**) Bar charts show numbers within each thymocyte population for the indicated strains.

Mice are 7-20 weeks old. Cre- (WT), (n = 17); $CD4^{Cre}$ $Rela^{fx/fx}$, (n = 23); $Nfkb1^{-/-}$, (n = 19); $CD4^{Cre}$ $Rela^{fx/fx}$ $Nfkb1^{-/-}$, (n = 17). For all density plots, numbers represent the percentage of cells in the corresponding gate. Bar charts show mean cell number ± SEM. ns = not significant; * = significant at $P<0.05$; ** = significant at $P<0.01$.

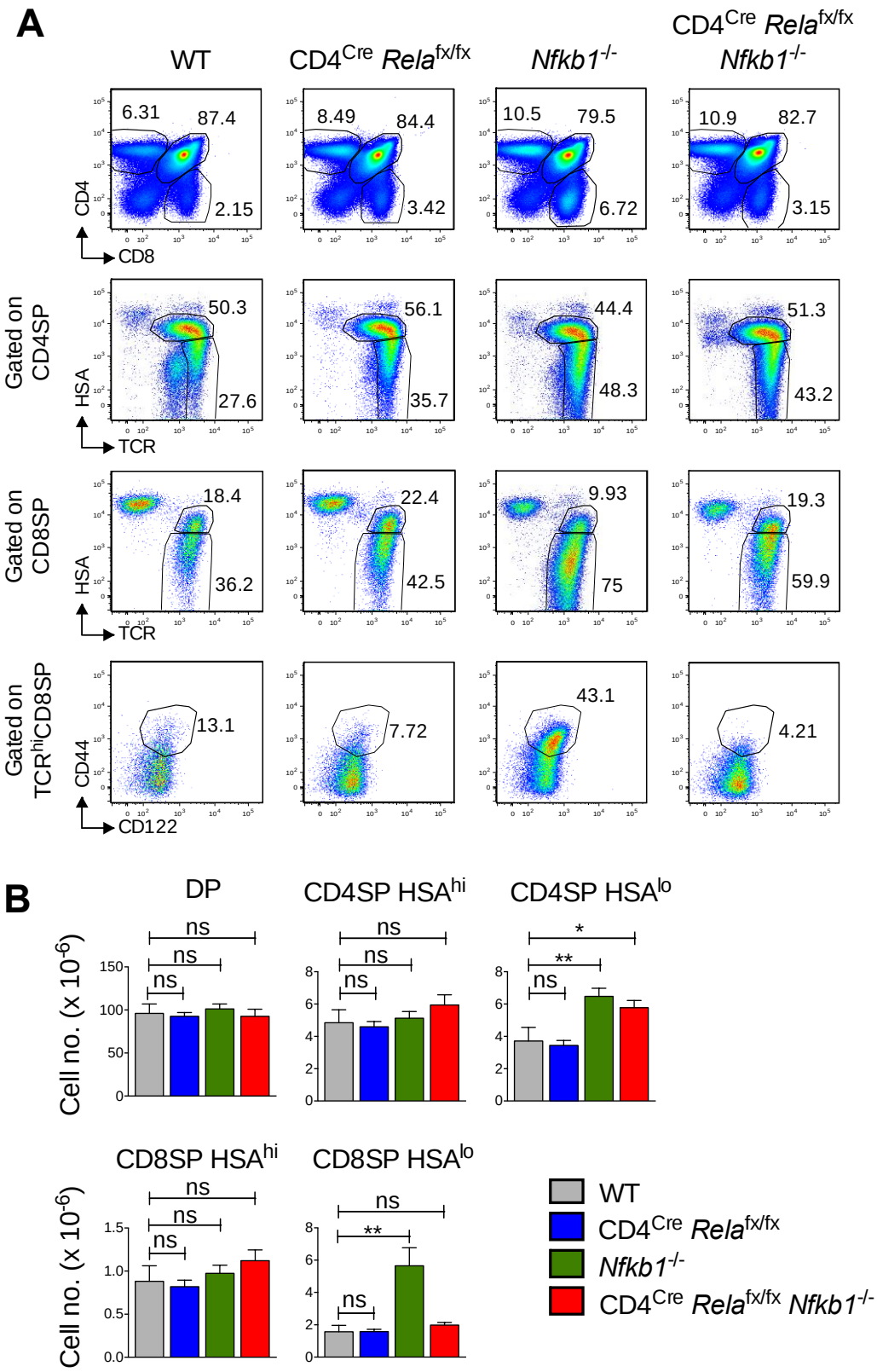

A
WT
CD4Cre Relafx/fx
Nfkb1-/-
CD4Cre Relafx/fx Nfkb1-/-
6.31
87.4
2.15
8.49
84.4
3.42
10.5
79.5
6.72
10.9
82.7
3.15
CD4
CD8
Gated on CD4SP
50.3
27.6
56.1
35.7
44.4
48.3
51.3
43.2
HSA
TCR
Gated on CD8SP
18.4
36.2
22.4
42.5
9.93
75
19.3
59.9
Gated on TCRhiCD8SP
13.1
7.72
43.1
4.21
CD44
CD122
B
DP
CD4SP HSAhi
CD4SP HSAlo
CD8SP HSAhi
CD8SP HSAlo
Cell no. (x 10-6)
ns
*
**
WT
CD4Cre Relafx/fx
Nfkb1-/-
CD4Cre Relafx/fx Nfkb1-/-

Figure 3.9 Mice with a deficiency of both NF-κB1 and RelA have fewer peripheral T cells

Mice with a conditional deficiency of RelA ($CD4^{Cre}$ $Rela^{fx/fx}$ $R26R^{EYFP}$) were crossed with mice in which NF-κB1 had been knocked out (*Nfkb1*$^{-/-}$) to produce mice deficient in both RelA and NF-κB1 ($CD4^{Cre}$ $Rela^{fx/fx}$ *Nfkb1*$^{-/-}$ $R26R^{EYFP}$). (**A**) Moving from top to bottom, density plots show: SSc vs TCR expression by total live lymphocytes; CD25 vs CD44 expression by $CD4^+$ T cells; TCR vs CD44 expression by $CD8^+$ T cells. (**B**) Combined T cell numbers within the LNs and spleen are shown for the indicated strains. (**C**) Within the LNs, naïve T cell populations were examined for their expression of the IL-7R. Bar charts show the mean fluorescence intensity (MFI) of IL-7R staining expressed as a percentage of that seen on "WT" cells (i.e. Cre- cells prepared, stained, and analysed concomitantly). Histograms underneath show the intensity of the fluorescent staining.

Mice are 7-20 weeks old. Cre- (WT), (n = 17); $CD4^{Cre}$ $Rela^{fx/fx}$, (n = 23); *Nfkb1*$^{-/-}$, (n = 19); $CD4^{Cre}$ $Rela^{fx/fx}$ *Nfkb1*$^{-/-}$, (n = 17). For all density plots, numbers represent the percentage of cells in the corresponding gate. Bar charts show mean ± SEM. ns = not significant; * = significant at $P<0.05$; *** = significant at $P<0.001$.

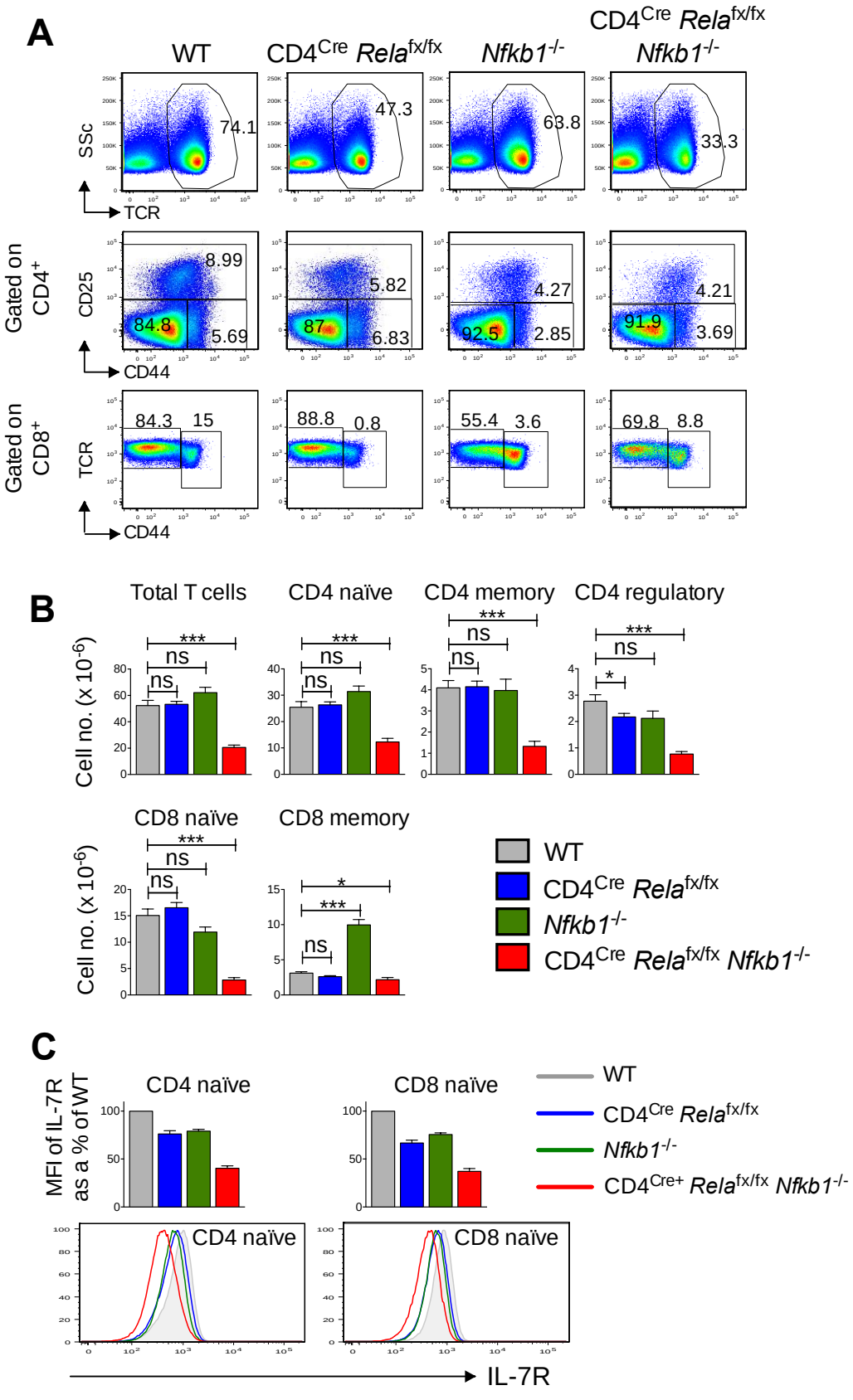

A
WT
CD4Cre Relafx/fx
Nfkb1-/-
CD4Cre Relafx/fx Nfkb1-/-
SSc
TCR
74.1
47.3
63.8
33.3
Gated on CD4+
CD25
CD44
8.99
84.8
5.69
5.82
87
6.83
4.27
92.5
2.85
4.21
91.9
3.69
Gated on CD8+
TCR
CD44
84.3
15
88.8
0.8
55.4
3.6
69.8
8.8
B
Total T cells
CD4 naïve
CD4 memory
CD4 regulatory
CD8 naïve
CD8 memory
Cell no. (x 10-6)
***
ns
*
WT
CD4Cre Relafx/fx
Nfkb1-/-
CD4Cre Relafx/fx Nfkb1-/-
C
MFI of IL-7R as a % of WT
CD4 naïve
CD8 naïve
WT
CD4Cre Relafx/fx
Nfkb1-/-
CD4Cre+ Relafx/fx Nfkb1-/-
IL-7R

Table 3.1 -Chapter 3 summary- Mouse T cell phenotypes

This table summarises the T cell phenotypes observed in the different strains of mice discussed in **Chapter 3**. The table refers to cell numbers among the different T cell subsets in the thymus and periphery (LN and spleen). An exception is made for IL-7R expression, whereby the table refers to the MFI of the IL-7R (as determined by FACS). For each mouse strain, the table takes into account total T cell numbers, both YFP+ and YFP-, within each T cell subset. Relative to wild-type mice, the T cell numbers and IL-7R MFI may either be "normal", increased (shown by one or more up arrows), or decreased (shown by one or more down arrows).

Lymphoid organs	T cell subset		huCD2^{iCre} *Ikk1*$^{fx/fx}$ (IKK1 deficient)	huCD2^{iCre} *Ikk2*$^{fx/fx}$ (IKK2 deficient)	IKKΔT^{CD4} (IKK1 & IKK2 deficient)	CD4^{Cre} *Rela*$^{fx/fx}$ (RelA deficient)	*Nfkb1*$^{-/-}$ (p105 and p50 deficient)	CD4^{Cre} *Rela*$^{fx/fx}$ *Nfkb1*$^{-/-}$ (RelA, p105 & p50 deficient)
Thymus	Total thymus		Normal	Normal	Normal	Normal	Normal	Normal
	DP		Normal	Normal	Normal	Normal	Normal	Normal
	CD4SP	HSA^{hi}	Normal	↑	Normal	Normal	Normal	Normal
		HSA^{lo}	Normal	Normal	↓↓↓	Normal	↑↑	↑
	CD8SP	HSA^{hi}	Normal	↑	↓↓↓	Normal	Normal	Normal
		HSA^{lo}	Normal	Normal	↓↓↓↓	Normal	↑↑↑	Normal
Lymph nodes & Spleen	Total T cells		Normal	↓↓	↓↓↓	Normal	Normal	↓↓
	CD4^{+}	Naïve	Normal	↓	↓↓↓↓	Normal	Normal	↓↓
		Memory	↓↓	↓↓↓	↓↓	Normal	Normal	↓↓
		Regulatory	↓↓	↓↓↓	↓↓	↓	Normal	↓↓
		IL-7R expression on naïve	Normal	↓↓	↓↓↓↓	↓	↓	↓↓
	CD8^{+}	Naïve	Normal	↓↓	↓↓↓↓	Normal	Normal	↓↓↓
		Memory	Normal	↓↓	↓	Normal	↑↑↑	↓
		IL-7R expression on naïve	↓	↓↓	↓↓	↓	↓	↓↓

Chapter 4 A role for TNF signalling in T cell development

4.1 Introduction

Inflammatory cytokines activate the canonical pathway of NF-κB signalling, typically resulting in the formation of RelA or c-Rel containing complexes (Lawrence, 2009). NF-κB may then drive transcription of genes encoding for cytokines, chemokines, and adhesion molecules; thus contributing towards a pro-inflammatory environment (Lawrence, 2009). The best-documented inflammatory cytokines that activate NF-κB are IL-1 and TNF. Both are pleiotropic cytokines that mediate a variety of biological functions.

TNF, as first described in the serum of bacillus Calmette Guérin infected mice, was found to have anti-tumour effects (Carswell et al., 1975). Today its name alone, “tumour necrosis factor”, reminds us of its cytotoxic abilities. However, in addition to cell death, TNF can mediate a variety of effects including cell survival and proliferation (Varfolomeev and Ashkenazi, 2004). There are two receptors for TNF, TNFR1 and TNFR2, and almost every cell type expresses one or the other of these (Brenner et al., 2015). TNF is produced as a homotrimer and initially expressed as a transmembrane protein, before being cleaved into its soluble form. Although both membrane-bound and soluble TNF serve to activate TNFR1, TNFR2 responds preferentially to the transmembrane form (Naudé et al., 2011).

TNFR1 is believed to be the main mediator of TNF induced signalling, since it is more widely expressed than TNFR2 (Naudé et al., 2011). Stimulation of each receptor results in initiation of somewhat different signalling cascades, but ultimately, ligation of either TNFR1 or TNFR2 can activate NF-κB. Although TNFR1 contains a death domain in the intracellular region, this is absent in TNFR2. The death domain of TNFR1 allows for recruitment of TNF receptor associated-protein with death domain (TRADD). TRADD can then recruit a

variety of other proteins, which allows for the formation of the two large complexes associated with TNFR1 signalling - complex I and complex II. Complex I is pro-survival. It enables recruitment of the IKK complex and leads to NF-κB activation. Complex II, however, results in caspase-8 activation and is usually pro-apoptotic. (Brenner et al., 2015). Occasionally, receptor-interacting protein kinase 3 (RIPK3) may can enter complex II, resulting in the formation of complex IIc (or the "necrosome") and leading to necroptosis (Han et al., 2011). The ultimate fate of a TNFR1 stimulated cell depends on the balance between the complex I mediated pro-survival and the complex II mediated cell-death pathways. TNFR2 lacks a death domain and cannot bind TRADD. Instead it may recruit TNFR associated factor 1 (TRAF1) and 2 (TRAF2) and activate NF-κB in this manner. TNFR2 cannot, however, initiate cell death (Brenner et al., 2015).

In the absence of NF-κB, many cells are sensitive to TNF induced death. This is perhaps best demonstrated in the case of knockout mouse strains. Mice with a complete deficiency of RelA, IKK2 or NEMO are all embryonic lethal due to massive apoptosis of fetal hepatocytes (Beg et al., 1995; Li et al., 1999b; 1999c; Rudolph et al., 2000). However, inactivation of the genes for either TNFR1 or TNF has been shown to rescue the lethal phenotype of the IKK2 and RelA knockout mice, respectively (Doi et al., 1999; Li et al., 1999b; Senftleben et al., 2001b). *In vitro* experiments have shown that fibroblasts, macrophages, B cells, and a variety of cell lines, lymphoid and otherwise, are all more sensitive to TNF induced death when deficient in NF-κB signalling (Beg and Baltimore, 1996; Schmidt-Supprian et al., 2000; 2003; Van Antwerp et al., 1996; Wang et al., 1996; Wu et al., 1996).

In **Chapter 3** we described the phenotype of IKKΔT^{CD4} mice. They were found to have a severe block between the HSA^{hi} and HSA^{lo} stages of SP thymocyte development. We hypothesised that the mechanism behind the block was TNF dependent. In the absence of NF-κB signalling, thymocytes, like many other cell types, are likely to be sensitive to TNF induced death. In **Chapter 4** we aimed to test this hypothesis by blocking TNF signalling in NF-κB deficient mice *in vivo*.

4.2 Results

4.2.1 *In vivo* blockade of TNF cytokine restores SP thymocyte development in IKKΔT^{CD4} mice

We wished to test the hypothesis that thymocytes have increased sensitivity towards TNF induced apoptosis in the absence of NF-κB signalling. We treated IKKΔT^{CD4} mice with intraperitoneal injections of either an anti-TNF blocking antibody (1mg per injection) or PBS, as control, on days 0, 2, and 4, before analysing hosts on day 7. CD4SP HSA^{lo}, CD8SP HSA^{hi} and CD8SP HSA^{lo} populations were all significantly reduced in the PBS treated IKKΔT^{CD4} mice as compared to the Cre- littermates (**Figure 4.1**). Remarkably, anti-TNF treatment over a one-week period restored thymocyte numbers to near WT levels. Numbers of CD8SP HSA^{lo} cells in the anti-TNF treated IKKΔT^{CD4} mice did, however, remain slightly lower than in WT mice (**Figure 4.1**). Treating the IKKΔT^{CD4} mice with blocking antibody over a two-week period (injections on days 0, 2, 4, 7, 9, and 11, followed by analysis on day 14) did not further increase SP numbers above those obtained by one week of treatment (**Figure 4.1B**).

Since the anti-TNF monoclonal antibody (mAb) was unlikely to be able to completely block TNF signalling, we bred IKKΔT^{CD4} mice onto a *Tnfrsf1a*$^{-/-}$ background. Importantly, *Tnfrsf1a*$^{-/-}$ mice, sufficient in NF-κB signalling, exhibit normal thymocyte development (**Figure 4.1**). Compared to the IKKΔT^{CD4} mice, the *Tnfrsf1a*$^{-/-}$ IKKΔT^{CD4} mice showed significant rescue of the CD4SP HSA^{lo}, CD8SP HSA^{hi} and CD8SP HSA^{lo} populations (**Figure 4.1**). In fact, the *Tnfrsf1a*$^{-/-}$ IKKΔT^{CD4} mice, as compared to their *Tnfrsf1a*$^{-/-}$ littermates, contained normal SP cell numbers, with the exception of the CD8SP HSA^{lo} cells, which remained slightly reduced (**Figure 4.1**). In conclusion, it appears that the reduction in SP thymocytes in IKKΔT^{CD4} mice can be attributed to death induced by TNF via TNFR1 dependent signalling.

4.2.2 *In vivo* blockade of TNF signalling in the IKKΔT^{CD4} mice causes a significant increase in naïve, peripheral T cells

We next wished determine if the restoration of SP development in IKKΔT^{CD4} mice also allowed the thymic emigration of new SP cells to fill the peripheral T cell compartment. We investigated the LNs and spleen of anti-TNF Ab or PBS treated IKKΔT^{CD4} mice. As compared to wild-type controls, the PBS treated IKKΔT^{CD4} mice had scarcely any peripheral T cells (**Figure 4.2**). After one week of anti-TNF Ab treatment, numbers of naïve cells in the IKKΔT^{CD4} mice were increased significantly. However, numbers failed to approach those observed in wild-type mice (**Figure 4.2B**). Anti-TNF Ab treatment had no apparent effect on numbers of regulatory or memory cells (**Figure 4.2**). Surprisingly, more prolonged treatment of mice with anti-TNF Ab for two weeks did not result in further increases in the naïve cell populations above those seen at the one week time point (**Figure 4.2B**).

Tnfrsf1a$^{-/-}$ mice had a normal peripheral T cell compartment with respect to numbers of naïve, memory, and regulatory cells (**Figure 4.2**). In comparison, *Tnfrsf1a*$^{-/-}$ IKKΔT^{CD4} mice had fewer naïve and regulatory cells (**Figure 4.2**). Compared to the IKKΔT^{CD4} mice, the *Tnfrsf1a*$^{-/-}$ IKKΔT^{CD4} mice showed significant rescue of the naïve cell pool. However, the extent of peripheral T cell rescue did not reach WT levels (**Figure 4.2**). In IKK deficient mice, the *in vivo* blockade of TNF signalling rescued developing T cells from death and allowed relatively normal thymic development and egress of new T cells. However, it seems that peripheral T cells in IKK deficient mice may have a reduced life span as compared to their wild-type counterparts, and this could not be corrected by blocking TNF activity.

4.2.3 TNF induced signalling is not essential for NF-κB mediated IL-7R upregulation on new T cells

NF-κB activation is necessary for normal expression of the IL-7R on naïve, peripheral T cells (**Chapter 3**; Miller et al., 2014; Silva et al., 2014). Recent

evidence suggests that members of the TNF receptor superfamily (TNFRSF) can induce IL-7R expression, in an NF-κB dependent way, on SP thymocytes (Silva et al., 2014). TNF was found to be particularly good at inducing IL-7R upregulation (Silva et al., 2014). Hence, we hypothesized that naïve T cells, which had experienced a lack of TNF signalling during development, may exhibit reduced IL-7R expression.

To test our hypothesis, we treated C57BL/6 wild-type mice with either anti-TNF blocking antibody or PBS control over a one-week period (according to the previously described protocol). Cell numbers in the thymus and periphery of the wild-type mice were not affected by the anti-TNF Ab treatment (**Figure 4.3A**; data not shown). Furthermore, expression of the IL-7R was not significantly reduced by the anti-TNF blockade (**Figure 4.3B, top**; **Figure 4.3C**). Next, we examined IL-7R levels on naïve T cells that lacked expression of TNFR1. IL-7R expression on the naïve T cells of *Tnfrsf1a*$^{-/-}$ mice was similar to that of wild-type controls (**Figure 4.3B, bottom**; **Figure 4.3C**). Therefore, it seems that TNF does not play an essential, non-redundant role in the normal upregulation of the IL-7R during T cell development.

We next examined IL-7R expression on the naïve T cells of *Tnfrsf1a*$^{-/-}$ IKKΔT^{CD4} mice and anti-TNF Ab treated IKKΔT^{CD4} mice. As established in **Chapter 3**, the CD4 naïve cells of IKKΔT^{CD4} mice have low IL-7R expression, whilst the CD8 naïve cells have levels near to those of WT cells. Interestingly, when the IKKΔT^{CD4} mice were either treated with anti-TNF blocking Ab or bred onto a TNFR1 knockout background, expression of the IL-7R on the CD8 naïve cells was further reduced (**Figure 4.3B, middle & bottom**; **Figure 4.3C**). Blocking TNF signalling in the IKK deficient mice has been shown to significantly increase the number of CD8 naïve cells (**Figure 4.2**). Furthermore, the percentage of cells expressing YFP increased greatly as a result of blocking TNF signalling (data not shown). This suggests that the naïve CD8 T cells detectable in IKKΔT^{CD4} mice had not completely deleted their *Ikk1* and *Ikk2* genes. By blocking TNF signalling with mAb or by deletion of TNFR1, the selection pressure for incomplete deletion (i.e. TNF induced death) was removed, allowing emergence of IKK deficient CD8 naïve T cells.

4.2.4 The TNF dependent block in SP thymocyte development is a result of reduced NF-κB activation

In murine embryonic fibroblasts, the IKK complex has been reported to prevent TNF induced death in an NF-κB independent manner (Yan et al., 2013). Therefore, it was important to determine whether or not the TNF induced death, observed in IKKΔT^{CD4} mice, was truly due to lack of NF-κB activation. pLck-IκB-PEST mice express a dominant negative form of IκBα under the control of the proximal Lck (pLck) promoter, resulting in a T cell specific inhibition of NF-κB activity (Voll et al., 2000). Mice expressing a super-inhibitory form of IκBα have a significant reduction in CD8SP thymocytes (Boothby et al., 1997; Esslinger et al., 1997; Hettmann and Leiden, 2000; Voll et al., 2000). Analysing the thymus of pLck-IκB-PEST mice revealed that the reduction in CD8SP numbers was actually due to loss of the more mature HSA^{lo} subset (**Figure 4.4**). We treated pLck-IκB-PEST mice with anti-TNF blocking Ab or PBS control for one week. Blocking TNF signalling in this manner completely restored the numbers of CD8SP HSA^{lo} thymocytes (**Figure 4.4**). Both IKKΔT^{CD4} mice and pLck-IκB-PEST mice exhibit a TNF dependent block in SP thymocyte maturation. Together, this suggests that the block in SP development is indeed due to a lack of NF-κB activation.

Assessment of the LNs and spleen of pLck-IκB-PEST mice revealed normal numbers of CD4^{+} cells, but a significant reduction in both the CD8 naïve and memory populations (**Figure 4.5A & B**). Treatment of the pLck-IκB-PEST mice with anti-TNF blocking Ab for one week failed to rescue peripheral T cell numbers (**Figure 4.5A & B**). Perhaps this is not surprising, as thymic output is unlikely to be sufficient over such a short time period to have a significant impact on the overall size of the peripheral pool.

The pLck-IκB-PEST mice have previously been described as having reduced expression of the IL-7R on their naïve T cells (Silva et al., 2014). We also found evidence of this: with IL-7R expression on the CD4 naïve cells reduced to approximately 36.8% (± 2.4%) of normal and expression on the CD8 naïve cells

reduced to just 28.7% (± 2.7%) of normal. Treatment of pLck-IκB-PEST mice with anti-TNF blocking Ab had no effect on IL-7R expression (**Figure 4.5C**).

4.2.5 *In vivo* blockade of TNF signalling fails to rescue IKK1 or IKK2 deficient B cells

We wondered whether the requirement for NF-κB in T cells reflected a more general requirement in developing lymphocytes. Therefore we chose to examine the effect of blocking TNF signalling on B cell development in IKK deficient mice. The huCD2 promoter and locus control region drive Cre mediated recombination of target genes from the DN2 stage of T cell development and very early in B cell development (de Boer et al., 2003). To investigate the effect of NF-κB deficiency in B cells we examined huCD2^{iCre} *Ikk1*$^{fx/fx}$ R26R^{EYFP} mice and huCD2^{iCre} *Ikk2*$^{fx/fx}$ R26R^{EYFP} mice. These strains have a deficiency in either IKK1 or IKK2 in both the T and B cell lineages.

Examining the bone marrow of huCD2^{iCre} *Ikk1*$^{fx/fx}$ R26R^{EYFP} mice and huCD2^{iCre} *Ikk2*$^{fx/fx}$ R26R^{EYFP} mice revealed that both strains had a severe reduction in the number of recirculating and mature B cells (**Figure 4.6**). Mature B cell numbers in the spleens of the IKK1 and IKK2 deficient mice were also reduced due to loss of both follicular and marginal zone populations (**Figure 4.6B**).

The block in T cell development in NF-κB deficient mice can be overcome by *in vivo* blockade of TNF signalling (**Figure 4.1**). We therefore hypothesized that the block in B cell development may also be TNF dependent. To test this we treated huCD2^{iCre} *Ikk1*$^{fx/fx}$ R26R^{EYFP} mice and huCD2^{iCre} *Ikk2*$^{fx/fx}$ R26R^{EYFP} mice with anti-TNF blocking Ab or PBS control over the course of one week. However, blocking TNF signalling had no effect on B cell development. The number of IgD^{+} mature cells in the bone marrow remained equally low, regardless of whether the mice had received anti-TNF or not (**Figure 4.6**). The recirculating population of B cells in the bone marrow was also unaffected by Ab treatment, as were mature B cell populations in the spleen (**Figure 4.6**). Since the defects in B cell development could not be overcome by TNF

blockade, we conclude that B cells have requirements for IKK activity that are distinct from T cells.

4.3 Discussion

In this chapter we tested the hypothesis that NF-κB activation protects developing T cells from TNF induced cell death. TNF signalling was blocked in IKKΔT^{CD4} mice, firstly by treatment with anti-TNF mAb and secondly by deletion of TNFR1. In both cases, SP thymocyte development was restored (**Figure 4.1**). In comparison, blocking TNF signalling in IKK sufficient mice had no noticeable effect. Wild-type mice treated with anti-TNF blocking Ab and *Tnfrsf1a*$^{-/-}$ mice both had normal numbers of SP thymocytes, showing no increase in cell number with respect to untreated, wild-type animals (**Figure 4.1; Figure 4.3A**). These results suggest that TNF is indeed killing SP thymocytes, but only when activity of the IKK complex is impaired (**Schematic 4.1**). In murine embryonic fibroblasts, the IKK complex has previously been reported to prevent TNF induced death in a manner independent of NF-κB (Yan et al., 2013). Therefore, we used pLck-IκB-PEST mice to further test our hypothesis that NF-κB activation protects thymocytes from TNF induced death. pLck-IκB-PEST mice have a block in NF-κB signalling at the level of IκBα degradation, as opposed to at the level of IKK complex formation. As with the IKKΔT^{CD4} mice, blocking TNF signalling in pLck-IκB-PEST mice also restored SP thymocyte development (**Figure 4.4**). Therefore, we are confident that NF-κB activation, mediated by IKK complex formation and IκBα degradation, is needed to protect developing T cells from TNF induced cell death.

Importantly, TNF induced death in the absence of NF-κB signalling does not appear to be a generalised lymphocyte feature. The bone marrow of huCD2^{iCre} *Ikk1*$^{fx/fx}$ R26R^{EYFP} mice and huCD2^{iCre} *Ikk2*$^{fx/fx}$ R26R^{EYFP} mice was found to contain reduced numbers of mature B cells (**Figure 4.6**). Blocking TNF signalling in these mice failed to rescue cell numbers (**Figure 4.6**). Hence, in IKK deficient mice, the block in B cell development, unlike that in T cell development, appeared to be independent of TNF.

IKKΔT^{CD4} mice had very few peripheral T cells. Initially, we hypothesised that the block in SP thymocyte development may alone account for the lack of

peripheral cells. Treating IKKΔT^{CD4} mice for one week with anti-TNF mAb did lead to a significant increase in naïve cells, but failed to restore peripheral T cell numbers to anywhere near wild-type levels (**Figure 4.2**). Thymic output was unlikely to be great over this short time period; therefore we also treated IKKΔT^{CD4} mice with mAb for two weeks. Surprisingly, treatment for two weeks did not further restore peripheral cell numbers beyond those of the one-week treated mice (**Figure 4.2**). Finally, we investigated the peripheral T cell compartment of *Tnfrsf1a*$^{-/-}$ IKKΔT^{CD4} mice. While total peripheral T cell numbers were greater in *Tnfrsf1a*$^{-/-}$ IKKΔT^{CD4} mice than anti-TNF Ab treated IKKΔT^{CD4} mice, they still remained below those of *Tnfrsf1a*$^{-/-}$ or WT controls (**Figure 4.2**). In IKK deficient mice, the loss of SP thymocytes can be attributed to TNF induced death. However, the loss of peripheral T cells is likely due to a combination of factors. Reduced thymic output and possibly also TNF induced death of naïve cells can only partially account for low peripheral cell numbers.

A lack of NF-κB signalling during T cell development results in the production of naïve cells that are deficient in IL-7R expression (**Chapter 3**; Miller et al., 2014; Silva et al., 2014). Since the maintenance of naïve cells depends heavily on IL-7 survival signals (Jameson, 2005), the decreased IL-7R expression among this population is likely to account for much peripheral cell loss. In addition, IKK2 deficient cells have been reported to show poor homeostatic proliferation (Schmidt-Supprian et al., 2004), and even a slight deficiency in NF-κB signalling has been show to reduce CD4^{+} memory and regulatory cell numbers (Chen et al., 2015; Schmidt-Supprian et al., 2003). We conclude that the lack of peripheral cells in our NF-κB signalling deficient mice is due to numerous factors.

In **Chapter 3** we described the generation of mixed bone marrow chimeras, whereby we reconstituted *Rag1*$^{-/-}$ hosts with bone marrow from congenically marked WT and IKKΔT^{CD4} mice. WT T cells showed normal development and peripheral expansion. However, the IKK deficient cells were blocked at the SP stage of thymocyte development – just as T cells in the intact IKKΔT^{CD4} mice had been. Importantly, this result showed us that the phenotype of the IKKΔT^{CD4} mice was not simply the product of unusually high TNF expression in

the thymus (a possibility, although TNF levels were not measured). When placed in a normal thymic environment the IKK deficient cells behaved exactly the same, whereas wild-type cells were unaffected.

Conventionally, macrophages are the largest producers of TNF (Chen and Goeddel, 2002) - although this may not be true in the thymic microenvironment. TNF production in the thymic medulla can be mediated by medullary thymic epithelial cells, conventional dendritic cells and plasmacytoid dendritic cells (Mahmud et al., 2014). Furthermore, in adult mice, evidence suggests that even the thymocytes themselves are producers of TNF (Giroir et al., 1992). In the periphery, TNF may be synthesized by activated $CD4^+$ and $CD8^+$ T cells (Chatzidakis and Mamalaki, 2010).

TNF has been found to induce IL-7R expression on developing T cells in an NF-κB dependent manner (Silva et al., 2014). This suggests a functional role for TNF in the thymus. However, naïve T cells that had matured in the complete absence of TNFR1 signalling were found to have normal levels of IL-7R expression (**Figure 4.3B & C**). Of note, TNF is not the only TNFSF member that can cause upregulation of the IL-7R (Silva et al., 2014). There is clearly potential for a large amount of redundancy among TNFSF members, hence the normal IL-7R expression in TNFR1 knockout mice and anti-TNF Ab treated WT mice (**Figure 4. 3B & C**).

NEMO is a key structural component of the IKK complex and an absence of NEMO, just like an absence of both IKK1 and IKK2, prevents NF-κB activation. Our IKK deficient mice closely phenocopy the previously described NEMO deficient mice (Schmidt-Supprian et al., 2003). This suggests that the block in SP development in the NEMO deficient animals may also be TNF dependent and may similarly be overcome by preventing signalling through TNFR1.

In response to TCR, TLR, or inflammatory cytokine signalling, the MAP3 kinase, TGFβ activating kinase 1 (TAK1), can contribute to the activation of the NF-κB and JNK pathways. It does this via phosphorylation of the IKK complex or MKK6 (Wang et al., 2001). Interestingly, mice with a T cell specific deficiency of

TAK1 share a similar phenotype to mice with a T cell specific deficiency of NEMO or of both IKK1 and IKK2. The TAK1 deficient mice have a severe reduction in numbers of SP thymocytes (largely due to loss of the HSA^{lo} subpopulations) and scarcely any peripheral T cells (Liu et al., 2006; Wan et al., 2006). The reduction in SP thymocytes was attributed to a block in TCR and IL-7 induced NF-κB activity in the absence of TAK1 expression (Wan et al., 2006). However, mice with a conditional loss of either IL-7 or TCR signalling at the DP stage of thymocyte development did not phenocopy the TAK1 deficient mice (McCaughtry et al., 2012; Sinclair and Seddon, 2014). We speculate that the phenotype of the TAK1 deficient mice is also due to TNF induced death.

Senftleben et al. have previously claimed a cell autonomous role for IKK2 in the protection of thymocytes from TNF induced apoptosis (Senftleben et al., 2001b). *Ikk2*$^{-/-}$ fetal liver stem cells were transferred into irradiated hosts. Six weeks later, thymic structure was found to be severely perturbed in the *Ikk2*$^{-/-}$ chimeras, with very few DN and DP cells being present and a complete absence of SP cells. However, co-transfer of wild-type bone marrow along with the *Ikk2*$^{-/-}$ stem cells reversed the defect, allowing IKK2 deficient T cells to develop and populate the periphery. It was suggested that the presence of the WT thymocytes prevented excess TNF production in the donor mice and that the IKK2 deficient cells were therefore protected from TNF induced apoptosis. *Ikk2*$^{-/-}$ *Tnfr1*$^{-/-}$ mice were then analyzed and found to have normal T cell development, which the authors claimed to further support a cell autonomous role for IKK2 in the protection from TNF (Senftleben et al., 2001b). The huCD2^{iCre} *Ikk2*$^{fx/fx}$ R26R^{EYFP} mice that we studied were found to have normal thymocyte development, suggesting that a deficiency of IKK2 from the DN2 stage of thymocyte development onwards does not render thymocytes susceptible to TNF induced death. We speculate that the lack of thymocytes in the *Ikk2*$^{-/-}$ radiation chimeras of Senftleben et al. could be due to TNF induced death of common lymphoid progenitors.

Mice expressing a lymphoid specific, dominant negative form of IκBα display variable phenotypes. The extent of the block in NF-κB signalling in these mice is transgene dose dependent: notably, animals expressing 80 copies of IκBα per

haploid chromosome complement have more severe phenotypes than those expressing 25 copies (Esslinger et al., 1997). The pLck-IκB-PEST mice we studied had a severe reduction in CD8SP cells in the thymus, but CD4SP cells were not significantly affected (**Figure 4.4**). This serves to further support the pre-existing idea that $CD8^+$ T cells have a greater requirement for NF-κB signalling than $CD4^+$ T cells (Boothby et al., 1997; Esslinger et al., 1997; Hettmann and Leiden, 2000; Schmidt-Supprian et al., 2003; Silva et al., 2014). Perhaps this is to be expected, given that CD8SP thymocytes have more transcriptionally active NF-κB than CD4SP thymocytes (Voll et al., 2000).

The reduction in the number of CD8SP thymocytes in mice expressing the dominant negative form of IκBα has previously been attributed to poor positive selection in the absence of NF-κB (Esslinger et al., 1997; Hettmann and Leiden, 2000; Jimi et al., 2008). However, through close examination of pLck-IκB-PEST mice, we found that the reduction in CD8SP numbers could largely be attributed to loss of the more mature HSA^{lo} subset, with the HSA^{hi} subset being hardly affected (**Figure 4.4**). This finding alone suggested that the CD8 lineage had undergone normal positive selection. Furthermore, by blocking TNF signalling in our pLck-IκB-PEST mice, we were able to completely restore the CD8SP population (**Figure 4.4**). Such evidence suggested that the reduction in CD8SP cells, seen in mice expressing the dominant negative form of IκBα, was due to TNF induced cell death in the absence of NF-κB signalling. As to whether NF-κB has any role to play in the positive selection of DP thymocytes remains a mystery, with more convincing evidence now required.

The development of a mature T cell requires a series of differentiation steps. High levels of NF-κB activation have long been reported in thymocytes, thereby suggesting a role for this transcription factor in T cell development (Voll et al., 2000). This concept is well supported by the phenotype of our $IKK\Delta T^{CD4}$ mice, in which T cell development is arrested at the late SP stage. Furthermore, it seems that TNF and other members of the TNFSF may play an NF-κB dependent role in the generation of new T cells (Silva et al., 2014). Signalling induced by ligation of the TNFR seems to provide a link to NF-κB activation and

subsequent induction of maturational cues, such as IL-7R upregulation (Silva et al., 2014).

From experiments performed in this chapter, we cannot definitively state the role of NF-κB in developing thymocytes. It seems that there are two possibilities. The first is that NF-κB signalling is needed to actively protect thymocytes from TNF induced death. The second is that NF-κB signalling is required at discrete stages during T cell development in order to produce a population of cells resistant to the cytotoxic effects of TNF. In **Chapter 5** we shall investigate whether an acute (as opposed to developmental) block in NF-κB signalling can render thymocytes sensitive to TNF induced death. Furthermore, the phenotype of our NF-κB deficient mice suggests that certain thymocyte subpopulations are more susceptible to the cytotoxic effects of TNF than others (**Figure 4.1**; **Figure 4.4**). This increased susceptibility may be cell intrinsic, or it may be due to the anatomical location of TNF production within the thymus. In **Chapter 5** we shall investigate this through the use of *in vitro* studies. Finally, we shall investigate the effect of TNF and other TNFSF members on thymocytes *in vitro*, in an attempt to determine possible physiological roles for these ligands in T cell development.

Figure 4.1 Blocking TNFR signalling in IKKΔT^{CD4} mice rescues thymocyte development

7-18 week old CD4^{Cre} *Ikk1*$^{fx/fx}$ *Ikk2*$^{fx/fx}$ R26R^{EYFP} (IKKΔT^{CD4}) mice were treated over a period of either 7 days or 14 days with a blocking antibody towards TNF or with PBS vehicle. Mice treated for 7 days received, in total, 3mg of antibody via three I.P. injections. Mice treated for 14 days received twice the amount of antibody and injections. At the end of treatment, animals were culled and analysed by FACS. Untreated, Cre- (WT) littermates were analysed in parallel. For a more complete block in TNFR signalling, IKKΔT^{CD4} mice were bred onto a TNFR1 knockout background. (**A**) T cell populations in the thymus were examined. Moving from top to bottom, density plots show: CD4 vs CD8 expression by TCR^{hi}CD5^{hi} thymocytes; HSA vs TCR expression by CD4SP thymocytes; HSA vs TCR expression by CD8SP thymocytes. (**B**) Total numbers of CD4SP and CD8SP thymocytes within the HSA^{hi} and HSA^{lo} gated populations are shown.

WT, (n = 17); IKKΔT^{CD4} PBS treated (n = 21); IKKΔT^{CD4} αTNF treated, 7 days, (n = 16); IKKΔT^{CD4} αTNF treated, 14 days (n = 8); *Tnfrsf1a*$^{-/-}$ (n = 13); *Tnfrsf1a*$^{-/-}$ IKKΔT^{CD4} (n = 16). Data are representative of a number of independent experiments. For all density plots, numbers represent the percentage of cells in the corresponding gate. Bar charts show mean cell number ± SEM. ns = not significant; * = significant at P<0.05; ** = significant at P<0.01; *** = significant at P<0.001.

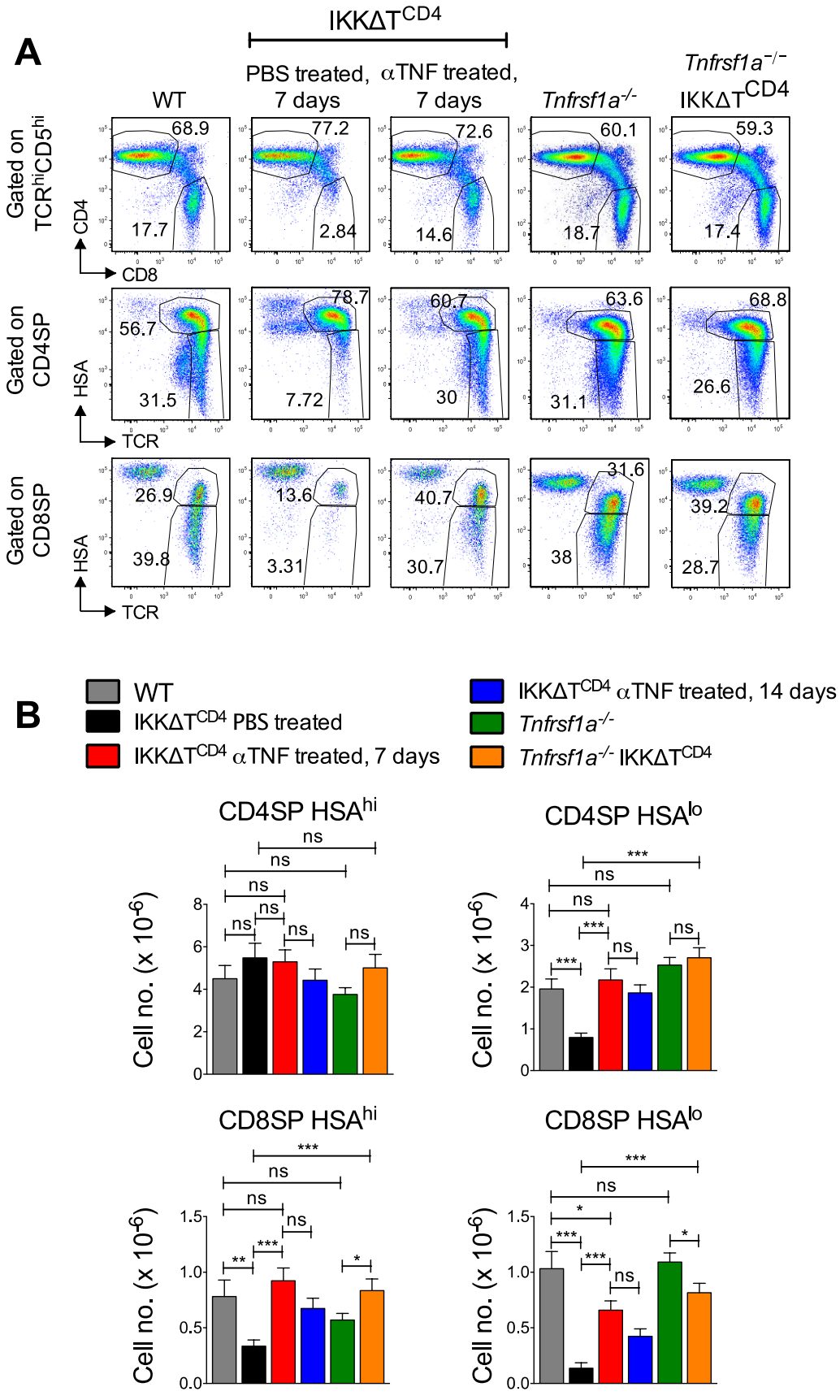

A
IKKΔT^CD4
PBS treated, 7 days
αTNF treated, 7 days
WT
Tnfrsf1a^-/-
Tnfrsf1a^-/- IKKΔT^CD4
Gated on TCR^hiCD5^hi
CD4
CD8
68.9
17.7
77.2
2.84
72.6
14.6
60.1
18.7
59.3
17.4
Gated on CD4SP
HSA
TCR
56.7
31.5
78.7
7.72
60.7
30
63.6
31.1
68.8
26.6
Gated on CD8SP
26.9
39.8
13.6
3.31
40.7
30.7
31.6
38
39.2
28.7
B
WT
IKKΔT^CD4 PBS treated
IKKΔT^CD4 αTNF treated, 7 days
IKKΔT^CD4 αTNF treated, 14 days
Tnfrsf1a^-/-
Tnfrsf1a^-/- IKKΔT^CD4
CD4SP HSA^hi
CD4SP HSA^lo
CD8SP HSA^hi
CD8SP HSA^lo
Cell no. (x 10^-6)
ns
***
**
*

Figure 4.2 Blockade of TNFR signalling in IKKΔT^{CD4} mice causes limited rescue of peripheral T cell numbers

7-18 week old CD4^{Cre} *Ikk1*$^{fx/fx}$ *Ikk2*$^{fx/fx}$ R26R^{EYFP} (IKKΔT^{CD4}) mice were treated over a period of either 7 days or 14 days with a blocking antibody towards TNF or with PBS vehicle. Mice treated for 7 days received, in total, 3mg of antibody via three I.P. injections. Mice treated for 14 days received twice the amount of antibody and injections. At the end of treatment, animals were culled and analysed by FACS. Untreated, Cre- (WT) littermates were analysed in parallel. For a more complete block in TNFR signalling, IKKΔT^{CD4} mice were bred onto a TNFR1 knockout background. (**A**) T cell populations in the LNs were examined. Density plots on top show CD5 vs TCR expression by total live lymphocytes. Density plots in the middle show CD25 vs CD44 expression by TCR^{hi}CD5^{hi}CD4^{+} cells. Density plots on the bottom show TCR vs CD44 expression by TCR^{hi}CD5^{hi}CD8^{+} cells. (**B**) Numbers of T cells in the periphery (LNs & spleen combined) were calculated for all mice.

WT, (n = 17); IKKΔT^{CD4} PBS treated (n = 21); IKKΔT^{CD4} αTNF treated, 7 days, (n = 16); IKKΔT^{CD4} αTNF treated, 14 days (n = 8); *Tnfrsf1a*$^{-/-}$ (n = 13); *Tnfrsf1a*$^{-/-}$ IKKΔT^{CD4} (n = 16). Data are representative of a number of independent experiments. For all density plots, numbers represent the percentage of cells in the corresponding gate. Bar charts show mean cell number ± SEM. ns = not significant; * = significant at P<0.05; ** = significant at P<0.01; *** = significant at P<0.001.

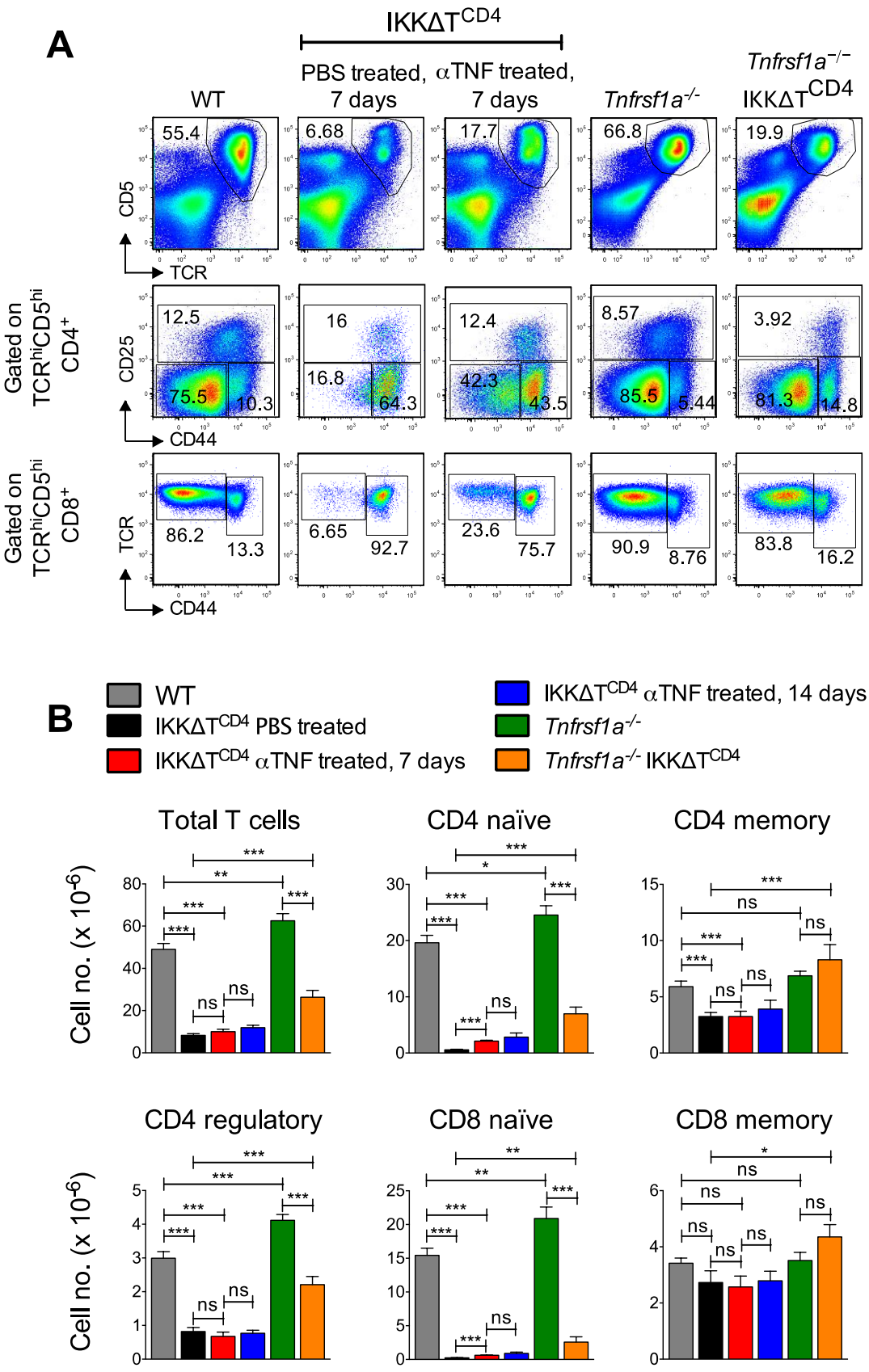

A
IKKΔT^CD4
PBS treated, 7 days
αTNF treated, 7 days
WT
Tnfrsf1a-/-
Tnfrsf1a-/- IKKΔT^CD4
CD5
TCR
CD25
CD44
Gated on TCRhiCD5hi CD4+
Gated on TCRhiCD5hi CD8+
55.4
6.68
17.7
66.8
19.9
12.5
75.5
10.3
16
16.8
64.3
12.4
42.3
43.5
8.57
85.5
5.44
3.92
81.3
14.8
86.2
13.3
6.65
92.7
23.6
75.7
90.9
8.76
83.8
16.2
B
WT
IKKΔT^CD4 PBS treated
IKKΔT^CD4 αTNF treated, 7 days
IKKΔT^CD4 αTNF treated, 14 days
Tnfrsf1a-/-
Tnfrsf1a-/- IKKΔT^CD4
Total T cells
CD4 naïve
CD4 memory
CD4 regulatory
CD8 naïve
CD8 memory
Cell no. (x 10-6)
ns

Figure 4.3 TNF induced signalling is not essential for NF-κB mediated IL-7R upregulation on new T cells

C57BL/6 (WT) mice were treated over a period of 7 days with either anti-TNF blocking Ab or PBS vehicle. $CD4^{Cre}$ *Ikk1*$^{fx/fx}$ *Ikk2*$^{fx/fx}$ $R26R^{EYFP}$ ($IKK\Delta T^{CD4}$) mice were treated for either 7 days or 14 days with anti-TNF blocking antibody or with PBS vehicle. Cre- (WT) littermates of $IKK\Delta T^{CD4}$ mice were left untreated, as were *Tnfrsf1a*$^{-/-}$ mice and *Tnfrsf1a*$^{-/-}$ $IKK\Delta T^{CD4}$ mice. (**A**) Bar charts show thymocyte numbers for the 7 day anti-TNF or PBS treated C57BL/6 (WT) mice. (**B**) Histograms show relative expression levels of the IL-7R on the naïve T cells within the LNs of the indicated mice. (**C**) The mean fluorescence intensity (MFI) of IL-7R staining on the naïve T cells within the LNs of the indicated mice was expressed as a percentage of that seen on “WT” cells (i.e. cells from untreated, WT mice prepared, stained, and analysed in parallel).

Mice were 10-23 weeks old upon analysis. C57BL/6 (WT) PBS treated, 7 days, (n = 4); C57BL/6 (WT) αTNF treated, 7 days, (n = 4); Cre- (WT), (n = 17); $IKK\Delta T^{CD4}$ PBS treated (n = 20); $IKK\Delta T^{CD4}$ αTNF treated, 7 days, (n = 16); $IKK\Delta T^{CD4}$ αTNF treated, 14 days (n = 8); *Tnfrsf1a*$^{-/-}$ (n = 13); *Tnfrsf1a*$^{-/-}$ $IKK\Delta T^{CD4}$ (n = 14). Data are representative of a number of independent experiments. Bar charts show mean ± SEM. ns = not significant.

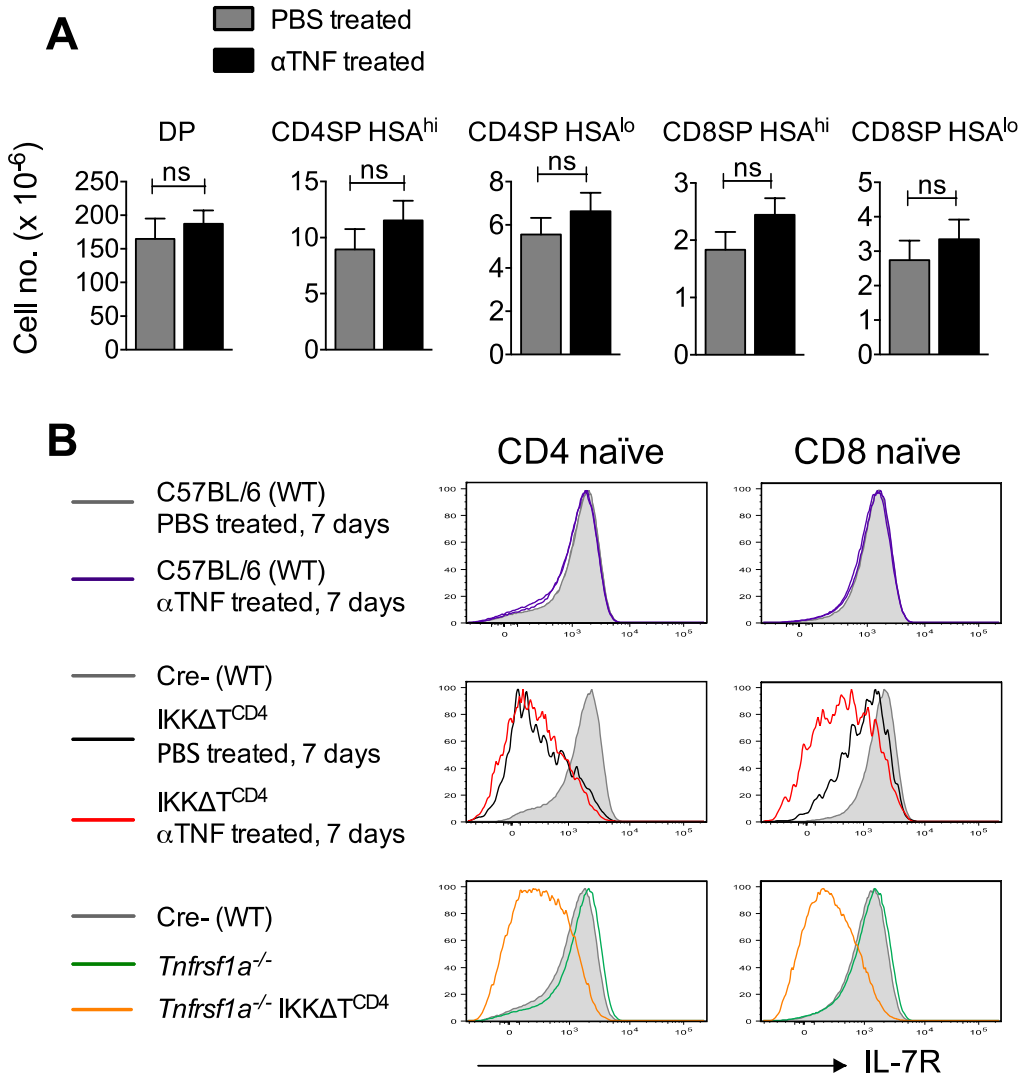

A
PBS treated
αTNF treated
DP
CD4SP HSAhi
CD4SP HSAlo
CD8SP HSAhi
CD8SP HSAlo
Cell no. (x 10-6)
ns
B
CD4 naïve
CD8 naïve
C57BL/6 (WT)
PBS treated, 7 days
C57BL/6 (WT)
αTNF treated, 7 days
Cre- (WT)
IKKΔTCD4
PBS treated, 7 days
IKKΔTCD4
αTNF treated, 7 days
Cre- (WT)
Tnfrsf1a-/-
Tnfrsf1a-/- IKKΔTCD4
IL-7R
C
Cre- (WT)
C57BL/6 (WT) αTNF treated, 7 days
IKKΔTCD4 PBS treated
IKKΔTCD4 αTNF treated, 7 days
IKKΔTCD4 αTNF treated, 14 days
Tnfrsf1a-/-
Tnfrsf1a-/- IKKΔTCD4
CD4 naïve
CD8 naïve
IL-7R MFI
as a % of WT

Figure 4.4 Mice expressing a super-inhibitory form of IκBα have a defect in the later stages of CD8SP thymocyte development, which may be overcome by blocking TNF

7-16 week old pLck-IκB-PEST mice, which express a super-inhibitory form of IκBα under the control of the pLck promoter, were treated for 7 days with an anti-TNF blocking antibody or with PBS control. Untreated WT littermates, lacking the IκBα super-inhibitor, were analysed in parallel. (**A**) From top to bottom, thymic profiles show: CD4 vs CD8 expression by total live thymocytes; CD5 vs TCR expression by total live thymocytes; CD4 vs CD8 expression by $TCR^{hi}CD5^{hi}$ thymocytes; HSA vs TCR expression by CD4SP thymocytes; HSA vs TCR expression by CD8SP thymocytes. (**B**) Cell numbers within the indicated thymocyte populations are shown.

WT, (n = 4); pLck-IκB-PEST, PBS treated (n = 8); pLck-IκB-PEST, αTNF treated (n = 8). The experiment was repeated twice with similar results. Bar charts represent data from both experiments. For all density plots, numbers represent the percentage of cells in the corresponding gate. Bar charts show mean cell number ± SEM. ns = not significant; * = significant at $P<0.05$; *** = significant at $P<0.001$.

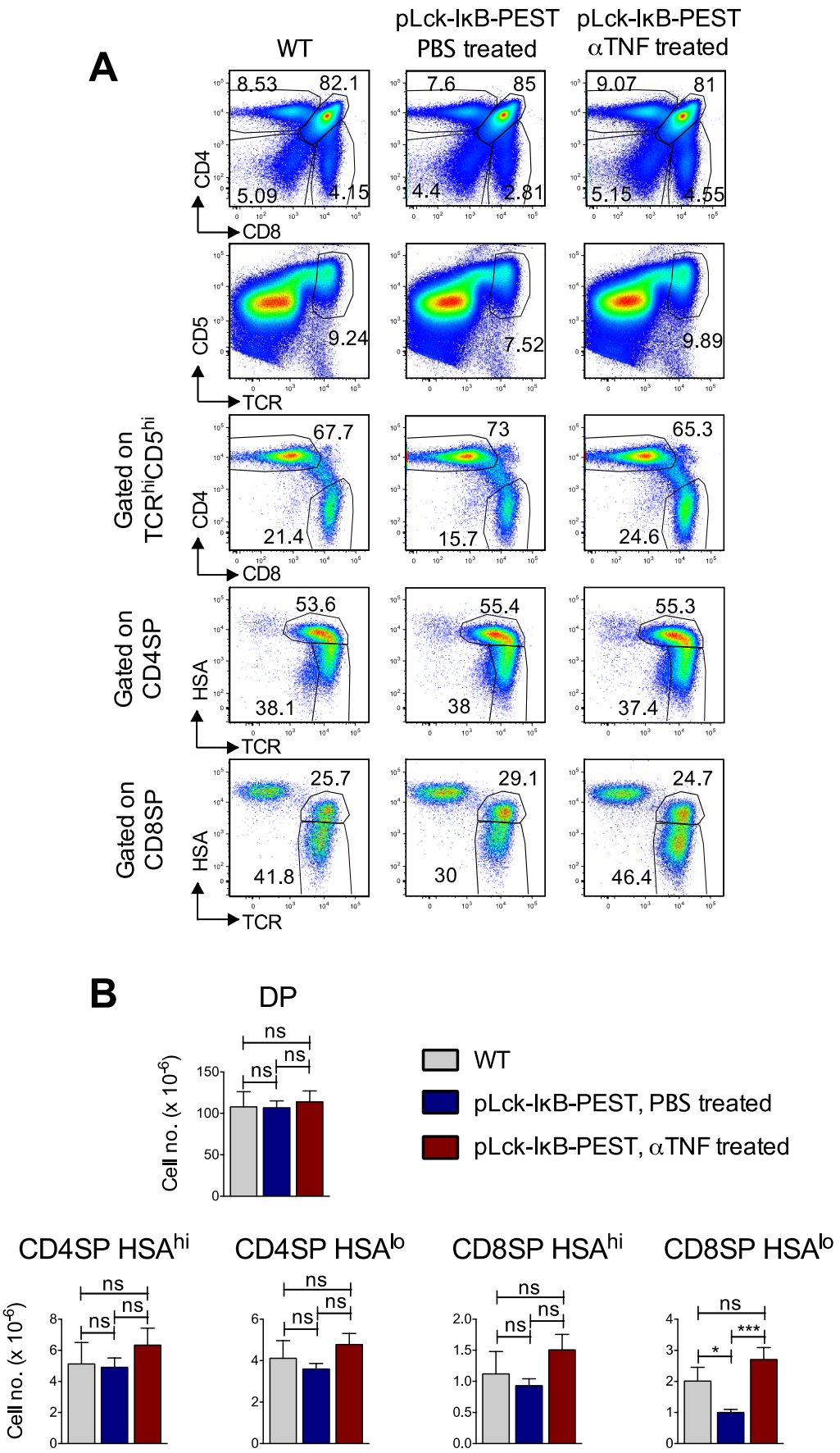

A
WT
pLck-IκB-PEST PBS treated
pLck-IκB-PEST αTNF treated
8.53
82.1
5.09
4.15
7.6
85
4.4
2.81
9.07
81
5.15
4.55
CD4
CD8
9.24
7.52
9.89
CD5
TCR
Gated on TCRhiCD5hi
67.7
21.4
73
15.7
65.3
24.6
53.6
38.1
55.4
38
55.3
37.4
Gated on CD4SP
HSA
25.7
41.8
29.1
30
24.7
46.4
Gated on CD8SP
B
DP
Cell no. (x 10-6)
ns
WT
pLck-IκB-PEST, PBS treated
pLck-IκB-PEST, αTNF treated
CD4SP HSAhi
CD4SP HSAlo
CD8SP HSAhi
CD8SP HSAlo
*
***

Figure 4.5 Blocking TNF signalling in mice expressing a super-inhibitory form of IκBα fails to restore peripheral T cell numbers

7-16 week old pLck-IκB-PEST mice, which express a super-inhibitory form of IκBα under the control of the pLck promoter, were treated for 7 days with an anti-TNF blocking antibody or with PBS control. Untreated WT littermates, lacking the IκBα super-inhibitor, were analysed in parallel. (**A**) From top to bottom, LN profiles show: CD5 vs TCR expression by total live lymphocytes; CD4 vs CD8 expression by $TCR^{hi}CD5^{hi}$ cells; CD25 vs CD44 expression by $CD4^+$ T cells; TCR vs CD44 expression by $CD8^+$ T cells. (**B**) Peripheral T cell numbers (LNs and spleen combined) within the indicated populations are shown. (**C**) The mean fluorescence intensity (MFI) of IL-7R staining on naïve T cells from the LNs of the indicated mice was expressed as a percentage of that seen on WT cells.

WT, (n = 5); pLck-IκB-PEST, PBS treated (n = 8); pLck-IκB-PEST, αTNF treated (n = 8). The experiment was repeated twice with similar results. Bar charts represent data from both experiments. For all density plots, numbers represent the percentage of cells in the corresponding gate. Bar charts show mean ± SEM. ns = not significant; * = significant at $P<0.05$; ** = significant at $P<0.01$; *** = significant at $P<0.001$.

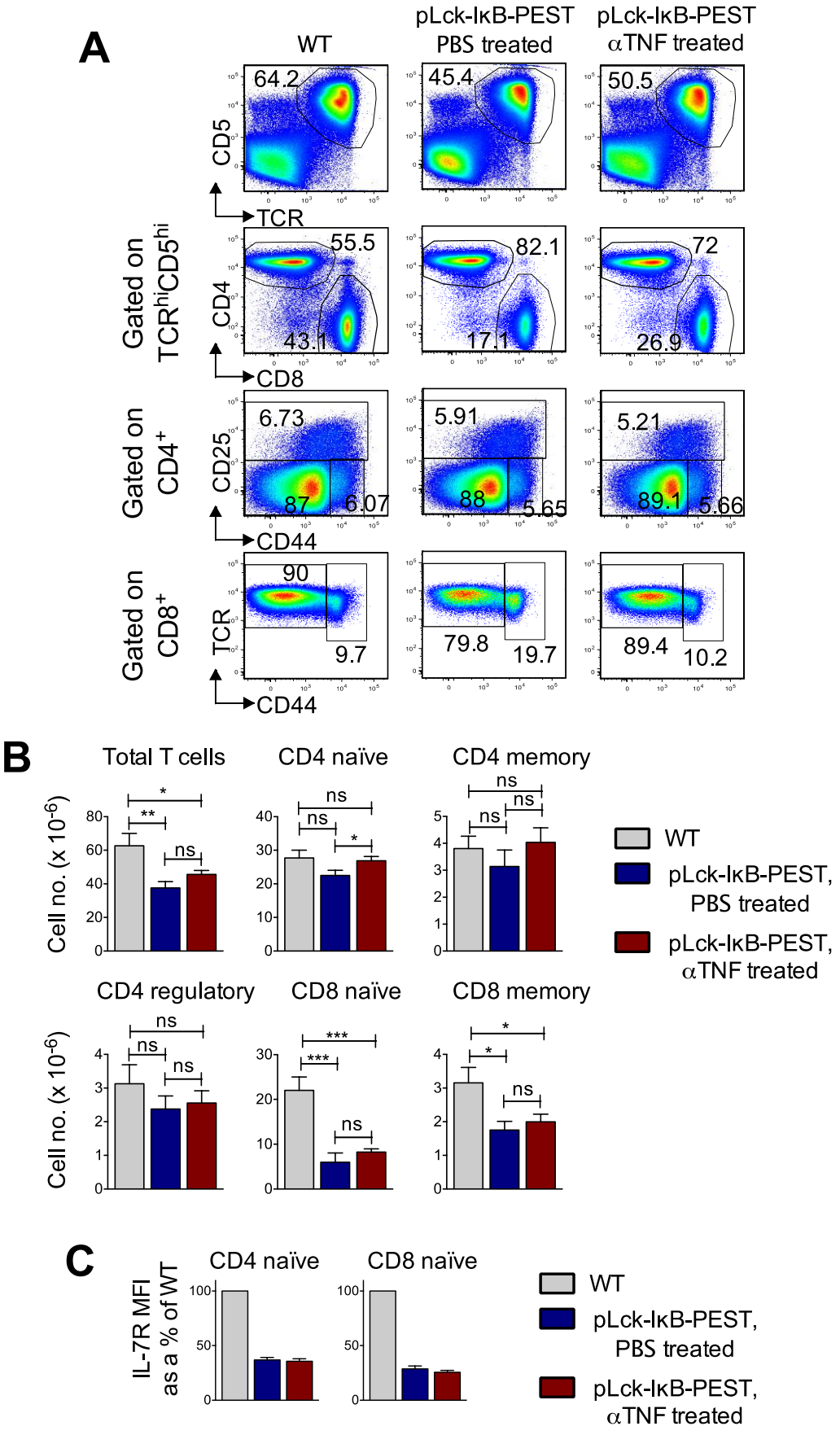
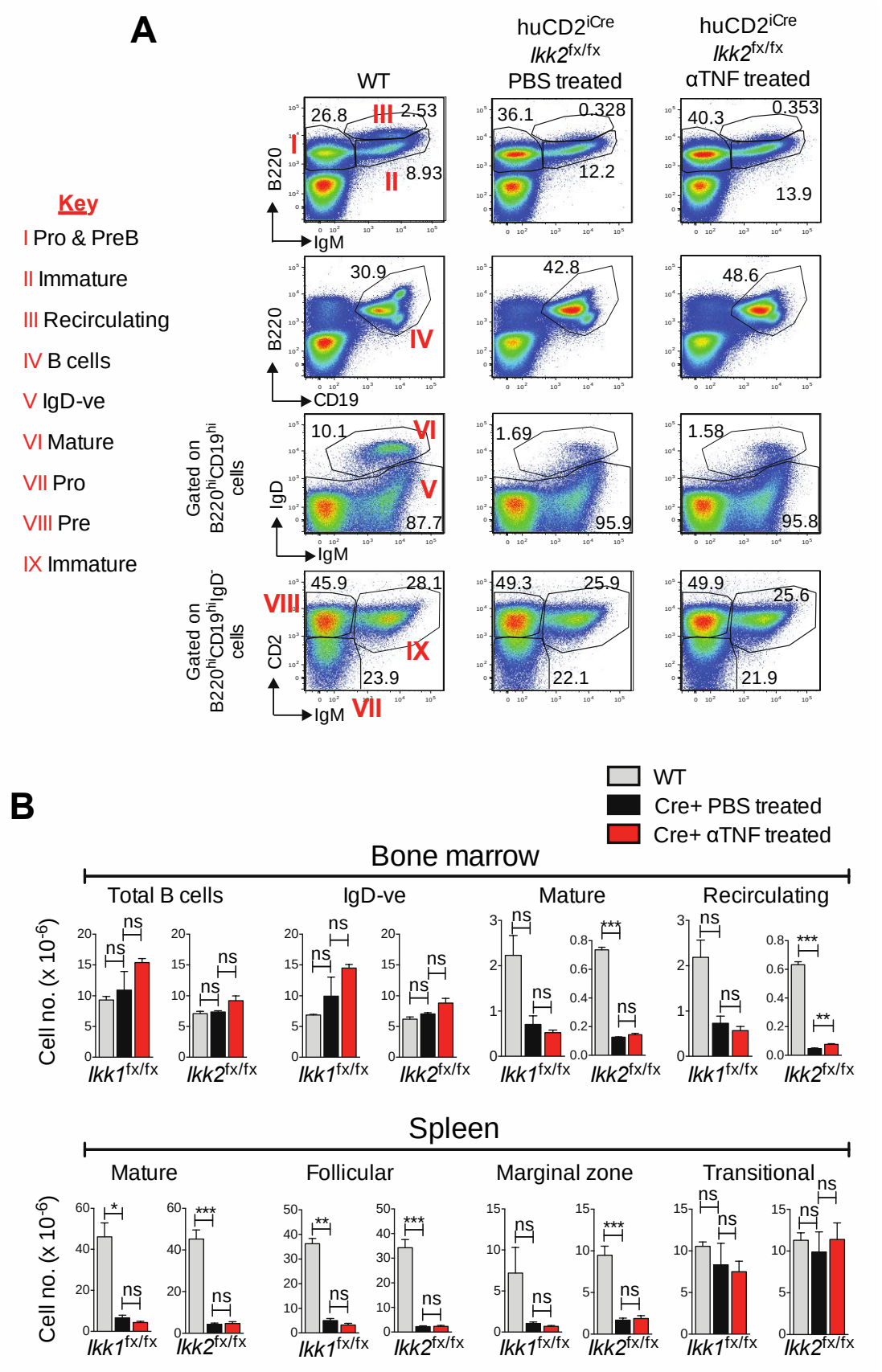

A
WT
pLck-IκB-PEST PBS treated
pLck-IκB-PEST αTNF treated
64.2
45.4
50.5
CD5
TCR
Gated on TCRhiCD5hi
55.5
43.1
82.1
17.1
72
26.9
CD4
CD8
Gated on CD4+
6.73
87
6.07
5.91
88
5.65
5.21
89.1
5.66
CD25
CD44
Gated on CD8+
90
9.7
79.8
19.7
89.4
10.2
TCR
CD44
B
Total T cells
CD4 naïve
CD4 memory
CD4 regulatory
CD8 naïve
CD8 memory
Cell no. (x 10-6)
ns
*
**
***
WT
pLck-IκB-PEST, PBS treated
pLck-IκB-PEST, αTNF treated
C
CD4 naïve
CD8 naïve
IL-7R MFI as a % of WT
100
50
0
WT
pLck-IκB-PEST, PBS treated
pLck-IκB-PEST, αTNF treated

Figure 4.6 TNF induced apoptosis cannot account for the block in B cell development in huCD2^{iCre} *Ikk1*$^{fx/fx}$ R26R^{EYFP} mice and huCD2^{iCre} *Ikk2*$^{fx/fx}$ R26R^{EYFP} mice

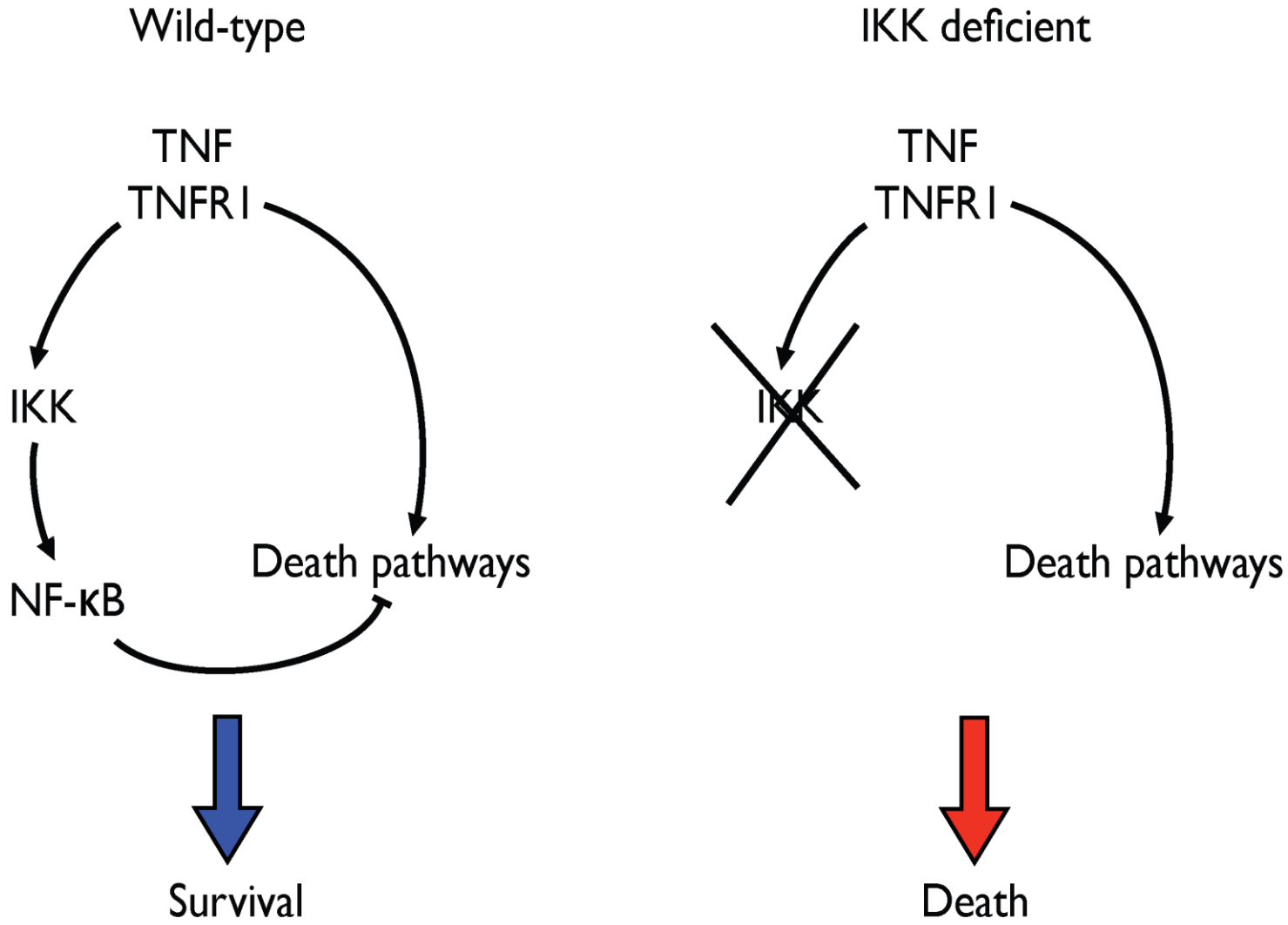
8 week old huCD2^{iCre} *Ikk1*$^{fx/fx}$ R26R^{EYFP} mice and 7 week old huCD2^{iCre} *Ikk2*$^{fx/fx}$ R26R^{EYFP} mice were treated for 7 days with an anti-TNF blocking antibody or with PBS vehicle. Cre- (WT) littermates were left untreated. B cell populations in the bone marrow and spleen were then assessed. (**A**) FACS plots show B cell populations in the bone marrow of huCD2^{iCre} *Ikk2*$^{fx/fx}$ R26R^{EYFP} mice treated with PBS or αTNF and an untreated Cre- littermate. From top to bottom, density plots show: B220 vs IgM expression by total live lymphocytes; B220 vs CD19 expression by total live lymphocytes; IgD vs IgM expression by B220^{hi}CD19^{hi} cells; CD2 vs IgM expression by IgD^{-} B cells. The key shows the different B cell populations within the bone marrow. (**B**) Bar charts show B cell numbers within the bone marrow (top) or spleen (bottom) of PBS and αTNF treated huCD2^{iCre} *Ikk1*$^{fx/fx}$ R26R^{EYFP} mice or huCD2^{iCre} *Ikk2*$^{fx/fx}$ R26R^{EYFP} mice and cell numbers in untreated Cre- (WT) littermates.

Cre- (WT) littermates of huCD2^{iCre} *Ikk1*$^{fx/fx}$ R26R^{EYFP} mice, (n = 2); huCD2^{iCre} *Ikk1*$^{fx/fx}$ R26R^{EYFP} PBS treated mice, (n = 2); huCD2^{iCre} *Ikk1*$^{fx/fx}$ R26R^{EYFP} αTNF treated mice, (n = 3); Cre- (WT) littermates of huCD2^{iCre} *Ikk2*$^{fx/fx}$ R26R^{EYFP} mice, (n = 3); huCD2^{iCre} *Ikk2*$^{fx/fx}$ R26R^{EYFP} PBS treated mice, (n = 3); huCD2^{iCre} *Ikk2*$^{fx/fx}$ R26R^{EYFP} αTNF treated mice, (n = 3). For all density plots, numbers represent the percentage of cells in the corresponding gate. Bar charts show mean cell number ± SEM. ns = not significant; * = significant at P<0.05; ** = significant at P<0.01; *** = significant at P<0.001.


A
WT
huCD2iCre lkk2fx/fx PBS treated
huCD2iCre lkk2fx/fx αTNF treated
Key
I Pro & PreB
II Immature
III Recirculating
IV B cells
V IgD-ve
VI Mature
VII Pro
VIII Pre
IX Immature
B220
IgM
CD19
IgD
CD2
Gated on B220hiCD19hi cells
Gated on B220hiCD19hiIgD- cells
26.8
2.53
8.93
36.1
0.328
12.2
40.3
0.353
13.9
30.9
42.8
48.6
10.1
87.7
1.69
95.9
1.58
95.8
45.9
28.1
23.9
49.3
25.9
22.1
49.9
25.6
21.9
B
WT
Cre+ PBS treated
Cre+ αTNF treated
Bone marrow
Total B cells
IgD-ve
Mature
Recirculating
Cell no. (x 10-6)
lkk1fx/fx
lkk2fx/fx
Spleen
Mature
Follicular
Marginal zone
Transitional
ns
*
**
***

Schematic 4.1 -Chapter 4 summary- A balance between TNF induced survival and death

In wild-type, mature SP thymocytes there is a fine balance between TNF induced production of pro-survival proteins and TNF induced activation of pro-death pathways. When NF-κB signalling is intact, TNF does not cause death of mature SP thymocytes. However, in IKK deficient, mature SP thymocytes TNF induced pro-death pathways cannot be inhibited and the cell dies.


Wild-type
TNF
TNFR1
IKK
NF-κB
Death pathways
Survival
IKK deficient
TNF
TNFR1
IKK
Death pathways
Death

Chapter 5 Activation of NF-κB signalling by the TNF receptor superfamily regulates thymocyte development

5.1 Introduction

NF-κB activation has a major role to play in proliferation, inflammation, and the prevention of apoptosis. For this reason, constitutive activation of the NF-κB signalling pathway has been held accountable for numerous human maladies, including cancer and chronic inflammatory diseases (Hayden and Ghosh, 2012). Much effort has been devoted to the development of NF-κB inhibitors and today over 700 are recognized (Gilmore and Herscovitch, 2006; Kwak et al., 2011). Despite the vast number of different inhibitors, they can be broadly classed into a few neat categories according to their mode of action. As Gilmore and Herscovitch explain: there are those that work upstream of the IKK complex (including those that block receptor signalling); those that work at the level of the IKK complex or IκB phosphorylation; those that prevent the degradation of IκB; those that inhibit the nuclear translocation of NF-κB; those that prevent the binding of NF-κB to DNA; and those that inhibit NF-κB dependent gene expression itself (Gilmore and Herscovitch, 2006). Due to its very central location in the NF-κB signalling pathway, and the fact that it acts to integrate numerous upstream signals, the IKK complex is a prime target for inhibition.

The ability of an inhibitor to block NF-κB signalling is usually measured by its IC_{50} value (the concentration of inhibitor required for a 50% inhibition of NF-κB activity) (Kwak et al., 2011). This is determined via tissue culture following stimulation of cells with a strong inducer of NF-κB activation, such as TNF, IL-1, or LPS (Gilmore and Herscovitch, 2006). Indeed, the fact that TNF is such a good activator of NF-κB makes it a potential target for inhibition in a number of NF-κB related diseases. For example, anti-TNF blocking antibodies are in current clinical use for the treatment of inflammatory bowel disease, Crohn's

disease, and arthritis (Gilmore and Herscovitch, 2006). In the clinic, the success of TNF inhibitors has generated recent interest in other TNF related cytokines and their receptors as potential clinical targets.

The tumour necrosis factor superfamily (TNFSF) consists of 19 cytokines, which interact with one or more of 29 receptors belonging to the tumour necrosis factor receptor superfamily (TNFRSF) (Aggarwal et al., 2012). The TNFSF members, in order of discovery, are: TNF (also known as TNF-α), TNF-β (also known as lymphotoxin-α), lymphotoxin-β, CD40L, FasL, CD30L, 4-1BBL, CD27L, OX40L, TRAIL, LIGHT, RANKL, TWEAK, APRIL, BAFF, TLA1, EDA-A1, EDA-A2, and GITRL (Aggarwal et al., 2012). All TNFSF members are pro-inflammatory, a function at least partially mediated by activation of NF-κB (Aggarwal et al., 2012). Other possible outcomes of TNFSF ligation include proliferation, angiogenesis, metastasis, morphogenesis, and apoptosis (Aggarwal et al., 2012). Blocking antibodies against many TNFSF ligands and receptors are in current clinical use. Anti-CD30 antibodies can be used in the treatment of cancer, anti-RANKL antibodies are administered in patients with osteoporosis, and anti-BAFF antibodies are believed to be beneficial in the treatment of systemic lupus erythematosus (SLE) (Croft et al., 2013).

TNFRSF members fall into two main categories – those that contain an intracellular death domain (DD) and those that do not (Aggarwal et al., 2012). The DD is normally composed of around 45 amino acids and acts to recruit various proteins that ultimately lead to the induction of cell death. Six TNFRSF members contain the DD: TNFR1 (DR1); Fas (DR2); DR3, which binds VEGI; DR4 and DR5, which bind TRAIL; and DR6, which binds no identified ligand (Aggarwal et al., 2012). The TNFSF is unusual in that many of its members are primarily expressed as transmembrane proteins rather than in their soluble forms. FasL, CD27L, CD30L, CD40L, OX40L, and 4-1BBL are all largely membrane associated (Aggarwal et al., 2012). For this reason they can engage in the act of “reverse signalling”, whereby a signal is transmitted from the receptor to the cell expressing the membrane bound ligand (Aggarwal et al., 2012).

Although the TNFSF has known functions in the cardiovascular, pulmonary, neurological, and metabolic systems, the highest expression of both ligands and receptors is found among cells of the immune system (Aggarwal et al., 2012). BAFF and APRIL both help to control the survival of peripheral B cells, and BAFF plays an additional role in B cell maturation (Mackay and Schneider, 2009). The receptor CD40 induces inflammatory cytokine production in macrophages and dendritic cells (Croft et al., 2013). LIGHT interacts with the HVEM receptor on T cells and increases the magnitude of a T cell response (Croft et al., 2013). TWEAK and its receptor help to enable interactions between immune and non-immune cells (Croft et al., 2013). In fact, every TNFSF member contributes, in some respect, to regulation of the immune system (Croft et al., 2013).

Perhaps unsurprisingly, TNFRSF members have been shown to be important in the thymus. Notably, DR5 deficient mice have enlarged thymii (Aggarwal et al., 2012). However, arguably the most well described thymic role for TNFRSF members is in the production of regulatory T cells. GITR, OX40, TNFR2, and CD27 have all been implicated in the development of thymic T_{reg} cells (Coquet et al., 2013; Mahmud et al., 2014). Ligands for all of these receptors are produced in the thymic medulla by dendritic cells and medullary epithelial cells (Coquet et al., 2013; Mahmud et al., 2014). Signalling via CD27 is important during the positive selection of thymic T_{reg} cells, when it acts to inhibit the mitochondrial apoptosis pathway (Coquet et al., 2013). Consequently, both CD27 deficient and CD27L deficient mice have reduced numbers of thymic and peripheral T_{reg} cells (Coquet et al., 2013). GITR, OX40, and TNFR2 are all upregulated on T_{reg} cells during their thymic development (Mahmud et al., 2014). Expression of these TNFRSF members by T_{reg} cells was found to correlate with the strength of signalling via the TCR (Mahmud et al., 2014). While deletion of GITR, OX40, or TNFR2 alone did not cause any reductions in T_{reg} cell numbers, the combined inhibition of all three prevented T_{reg} cell development (Mahmud et al., 2014). This serves to highlight the biological redundancy that exists among the TNFSF.

In **Chapter 4** we established that our IKKΔT^{CD4} mice had few HSA^{lo} SP thymocytes due to the TNF induced death of this population. TNF is present in the thymus, and evidence suggests that it does play a role in T cell development (Chatzidakis and Mamalaki, 2010; Mahmud et al., 2014; Silva et al., 2014). In this chapter we investigated the effects of TNF and other TNFSF members on SP thymocytes *in vitro*. In **Chapter 4** we used a purely genetic approach to inhibit NF-κB signalling in T cells *in vivo*. In this chapter we used a combined genetic and pharmacological approach in order to acutely block NF-κB signalling in thymocytes *in vitro*. This enabled us to determine whether or not NF-κB was actively required for the protection of SP thymocytes from TNF induced death. Furthermore, *in vitro* experiments allowed us to investigate whether certain SP subsets were intrinsically more sensitive to TNF induced death than others.

5.2 Results

5.2.1 Mature thymocytes from IKKΔT^{CD4} mice are susceptible to TNF induced death *in vitro*

From the phenotype of the intact IKKΔT^{CD4} mice, it appeared that certain thymocyte populations might be more susceptible to TNF induced death than others. We hypothesized that the increased susceptibility may either be cell intrinsic or may be due to the location of TNF production within the thymus. To investigate this we used an *in vitro* approach. Suspensions of whole, mashed thymii were cultured for 24hr in the presence of 30ng/mL recombinant murine TNF (**Figure 5.1A**). This removed any anatomical restrictions, thereby ensuring equal availability of the different thymocyte populations to TNF cytokine.

IKKΔT^{CD4} mice have very few SP thymocytes, which we attributed to TNF induced death. We were therefore concerned that the small number of remaining SP cells in the IKKΔT^{CD4} mice would show an uncharacteristically high resistance to TNF induced death, not representative of the population as a whole. As previously noted, *in vivo* treatment with anti-TNF Ab rescued the SP thymocyte populations in the IKKΔT^{CD4} mice. Therefore, prior to cell culture, IKKΔT^{CD4} mice and Cre- littermate controls were treated with anti-TNF blocking Ab over a period of 7 days. Thymocytes from the treated mice were then cultured for 24hr in the presence of PBS, TNF, or IL-7 (**Figure 5.1A**). The WT DP and CD4SP HSA^{hi} populations underwent significantly more death in the presence of TNF, as compared to PBS vehicle (**Figure 5.1A**). Addition of IL-7 reduced the level of cell death in all WT SP populations (**Figure 5.1A**). Treatment of the donor WT mice with anti-TNF blocking Ab prior to cell culture did not alter the subsequent response of thymocytes to either IL-7 or TNF *in vitro* (**Figure 5.1A**).

DP and CD4SP HSA^{hi} thymocytes from anti-TNF Ab treated IKKΔT^{CD4} mice were no more susceptible to TNF induced death than their WT counterparts. However, whilst CD4SP HSA^{lo}, CD8SP HSA^{hi} and CD8SP HSA^{lo} cells from WT

thymii were resistant to TNF, these same populations from anti-TNF Ab treated IKKΔT^{CD4} donors were highly sensitive to TNF induced cell death (**Figure 5.1A**). Culture with IL-7 failed to increase the viability of SP cells from the anti-TNF Ab treated IKKΔT^{CD4} donors (**Figure 5.1A**). Of note, the CD8SP HSA^{lo} cells from the anti-TNF Ab treated IKKΔT^{CD4} mice showed a high level of background death when cultured in medium supplemented only with PBS vehicle (**Figure 5.1A**).

Thymii from PBS treated IKKΔT^{CD4} mice were also cultured and compared to thymii from their anti-TNF Ab treated counterparts. Whether the NF-κB deficient mice had been anti-TNF treated or not, a similar pattern of cell death was apparent (**Figure 5.1A**). However, in response to TNF, the CD4SP HSA^{lo}, CD8SP HSA^{hi}, and CD8SP HSA^{lo} cells from the anti-TNF treated IKKΔT^{CD4} mice did show a slightly greater induction of cell death (**Figure 5.1A**). This supports the view that the few remaining thymocytes of these subsets in the PBS treated IKKΔT^{CD4} mice are enriched for cells that are TNF resistant.

As additional controls, thymii from *Tnfsf1a*$^{-/-}$ mice and *Tnfrsf1a*$^{-/-}$ IKKΔT^{CD4} mice were cultured in the presence of medium containing PBS, TNF, or IL-7. As expected, TNF failed to induce a change in viability in any of the populations from TNFR1 deficient donors (**Figure 5.1A**). IL-7 increased viability among the SP cells from *Tnfsf1a*$^{-/-}$ mice, but had no effect on the SP cells from *Tnfrsf1a*$^{-/-}$ IKKΔT^{CD4} mice (**Figure 5.1A**).

5.2.2 Subsets of DP thymocytes exhibit similar susceptibility to TNF induced death

As previously described, DP thymocytes can be further categorized into DP1-3 populations (Saini et al., 2010). DP1 cells (TCR^{lo}CD5^{lo}) are pre-selection, whilst DP2 cells (TCR^{int}CD5^{hi}) are in the early stages of selection into the CD4 and CD8 lineages, and DP3 cells (TCR^{hi}CD5^{int}) are in the later stages of selection into the CD8 lineage (Saini et al., 2010). Among WT thymii, total DP cells and CD4SP HSA^{hi} cells are weakly susceptible to TNF induced death, whereas

CD8SP HSA^{hi} cells are resistant (**Figure 5.1A**). Since CD4SP HSA^{hi} cells originate from the DP2 population and CD8SP HSA^{hi} cells originate from the DP3 population, we hypothesized that a thymocyte's susceptibility to TNF may well decrease between the DP2 and DP3 stages. To investigate this, we cultured WT thymocytes for 24hr in the presence of either 30ng/mL TNF or PBS vehicle and measured cell death among the different DP populations. However, over the course of a 24hr culture period, DP cells show substantial loss of their TCR and CD5 surface markers that hinder the identification of the different DP subsets (**Figure 5.1B, FACS plots**). Therefore we isolated purified populations of DP thymocytes by cell sorting. Prior to culture, the purity of the sorted cell populations was confirmed (**Figure 5.1B, top FACS plots**). All three DP subsets were found to have a similar, weak susceptibility to TNF induced death (**Figure 5.1B, bar charts**).

5.2.3 IκB super-repressor expression renders CD8SP HSA^{lo} cells sensitive to TNF induced death

pLck-IκB-PEST mice express a dominant negative IκBα and have reduced numbers of CD8SP HSA^{lo} thymocytes. In **Chapter 4** we showed that this deficiency could be rescued by *in vivo* blocking of TNF signalling. This suggests that, at physiological concentrations of TNF, only the CD8SP HSA^{lo} population is affected, and also that it is likely to be the most TNF sensitive subset. To confirm this, we cultured cell suspensions from whole thymii of pLck-IκB-PEST mice or transgene negative littermates with increasing concentrations of TNF cytokine (**Figure 5.2**). CD4SP HSA^{lo}, CD8SP HSA^{hi}, and CD8SP HSA^{lo} thymocytes from WT mice showed remarkable resistance to even the highest TNF concentrations. However, the CD4SP HSA^{hi} subset of WT thymocytes did show weak sensitivity to TNF induced cell death (**Figure 5.2**). The lowest concentrations of TNF were enough to induce death among this CD4SP HSA^{hi} population, and death was not further increased by use of higher TNF concentrations (**Figure 5.2**).

All SP thymocyte populations from pLck-IκB-PEST mice showed considerable sensitivity to the highest concentrations of TNF (**Figure 5.2**). Notably, CD4SP HSA^{hi} cells showed a similar amount of TNF induced death regardless of whether they were from the pLck-IκB-PEST mice or the WT mice (**Figure 5.2**). CD8SP HSA^{lo} cells from the pLck-IκB-PEST mice did exhibit a higher background level of death than the WT cells. However, even the lowest concentration of TNF used (0.1ng/mL) was enough to induce significant death in the IκBα super-repressor containing CD8SP HSA^{lo} population, whilst higher concentrations were required for induction of death among all other populations (**Figure 5.2**). In conclusion, among the pLck-IκB-PEST mice, the CD8SP HSA^{lo} population is indeed the most intrinsically sensitive to TNF.

5.2.4 Acute blockade in NF-κB signalling renders WT thymocytes susceptible to TNF induced death

Our previous results had indicated that NF-κB was required to protect thymocytes from TNF induced cell death. In $IKK\Delta T^{CD2}$ or $IKK\Delta T^{CD4}$ strains, deletion of IKK components occurred as early as the DN2 thymic stage, and yet it was mature SPs that were most susceptible to TNF induced cell death. In these strains, it was unclear whether NF-κB activity was 1) required to actively protect thymocytes from TNF induced death or 2) required at discrete developmental stages in order to programme cells to develop that are resistant to TNF induced cell death. To investigate these distinct possibilities, we took advantage of BI605906, a pharmacological and selective inhibitor of IKK2 (Clark et al., 2011). This enabled acute blockade of NF-κB signalling in thymocytes that had otherwise had NF-κB signalling intact during normal development.

As discussed in **Chapter 3**, IKK1 deficient mice show normal thymocyte development, other than a slight reduction in T_{reg} cells. It seems that canonical NF-κB signalling remains largely unperturbed in these mice, most likely due to the formation of IKK2 homodimers that can compensate for the loss of the IKK1 subunit. Addition of the IKK2 inhibitor, BI605906, to IKK1 deficient thymocytes

in vitro would be expected to prevent formation of the compensatory IKK2 homodimers and therefore cause a complete and acute block in NF-κB signalling.

Thymii from WT or huCD2^{iCre} *Ikk1*$^{fx/fx}$ R26R^{EYFP} mice were cultured with either 10μM BI605906 (IKK2 inhibitor) or DMSO vehicle, plus increasing concentrations of TNF cytokine (**Figure 5.3A**). When cultured with DMSO vehicle, only the CD4SP HSA^{hi} population of WT or IKK1 deficient thymocytes showed any TNF induced death. This was at a low level, as previously observed (**Figure 5.3A**; **Figure 5.1A**; **Figure 5.2**). In contrast, in the presence of the IKK2 inhibitor, TNF induced a high level of cell death in cultures. TNF induced higher levels of cell death in cultures of IKK1 deficient cells plus IKK2 inhibitor than in similar cultures of WT cells (**Figure 5.3A**). Interestingly, the IKK2 inhibitor did not increase cell death among the WT CD4SP HSA^{hi} population (**Figure 5.3A**).

Nonlinear regression was performed on the data collected from the huCD2^{iCre} *Ikk1*$^{fx/fx}$ R26R^{EYFP} cells treated with IKK2 inhibitor (**Figure 5.3B**). This enabled calculation of the IC_{50} values for the different SP subsets. The IC_{50} values revealed the concentrations of TNF required to increase cell death in each of the SP subsets to 50% of the maximum death observed. This revealed that CD8SP cells were most sensitive to TNF induced death, and that the CD8SP HSA^{lo} cells were the most sensitive subset of CD8SPs (**Figure 5.3B**).

5.2.5 *In vitro* stimulation with TNF induces IL-7R expression by SP thymocytes in an NF-κB dependent manner

In vitro stimulation with TNF cytokine has been shown to induce IL-7R expression on TCR transgenic CD8SP thymocytes (Silva et al., 2014). TNF induced IL-7R upregulation was reduced when cells were cultured in the presence of BI605906, or if the cells had an intrinsic deficiency of IKK2 (Silva et al., 2014). We wished to determine whether TNF could also induce IL-7R expression on the SP thymocytes of WT, polyclonal mice in an NF-κB

dependent manner. Single cell suspensions of thymii were obtained from WT mice and cultured for 24hr in the presence of TNF cytokine or PBS vehicle. Addition of TNF was found to increase IL-7R expression on some SP thymocyte subsets. The CD4SP HSA^{hi} cells showed the lowest increase in IL-7R expression, whilst the CD8SP HSA^{hi} cells showed the highest increase (**Figure 5.4A, top histograms**).

To confirm that TNF induced IL-7R expression was NF-κB dependent we cultured $IKK\Delta T^{CD4}$ thymocytes with TNF. Since $IKK\Delta T^{CD4}$ mice have few HSA^{lo} SP cells, we first treated $IKK\Delta T^{CD4}$ donors with anti-TNF blocking Ab over the course of one week. As before, this increased the number of SP thymocytes. Thymocyte suspensions from the treated $IKK\Delta T^{CD4}$ donors were then cultured for 24hr in the presence of TNF or PBS vehicle. TNF failed to induce IL-7R upregulation on any of the SP populations from the $IKK\Delta T^{CD4}$ mice (**Figure 5.4A, bottom histograms**).

We wished to confirm that the failure of the $IKK\Delta T^{CD4}$ cells to undergo TNF induced upregulation of the IL-7R was not due to the presence of residual anti-TNF blocking Ab in the culture wells. Therefore, we treated WT mice with anti-TNF blocking Ab or PBS vehicle for one week. As before, thymocyte suspensions were then washed before being cultured with TNF or PBS for 24hr. SP thymocyte populations from WT mice that had undergone the anti-TNF Ab treatment showed no inhibition in their upregulation of the IL-7R (**Figure 5.4B**). This indicated that anti-TNF blocking Ab was not influencing the cell culture. It seems that the failure of the $IKK\Delta T^{CD4}$ cells to upregulate IL-7R expression was indeed cell intrinsic.

5.2.6 Members of the TNFSF induce IL-7R expression on SP thymocytes

Notably, TNF does not account for all the NF-κB dependent regulation of IL-7R, since *Tnfsf1a*$^{-/-}$ mice showed normal IL-7R expression. Furthermore, it seems that TNF does not account for all the induced death among the IKK deficient,

CD8SP HSA^{lo} population, since blocking TNF signalling in IKKΔT^{CD4} mice could not completely restore CD8SP HSA^{lo} numbers. We concluded that there must be other ligands regulating both death and IL-7R induction among SP thymocytes.

To further investigate the NF-κB dependent regulation of death and IL-7R expression in thymocytes, we made use of F5 *Rag1*$^{-/-}$ (F5) thymocytes. F5 *Rag1*$^{-/-}$ mice produce a monoclonal population of CD8^{+} T cells expressing a transgenic, MHC class I restricted TCR specific for the NP68 influenza antigen (Mamalaki et al., 1993). F5 cells fail to upregulate the IL-7R after positive selection, resulting in a lack of expression during the SP stage of development (**Schematic 5.1**). This failure to initiate IL-7R expression is thought to be a consequence of suboptimal TCR signalling by the transgenic TCR during positive selection (Sinclair et al., 2011). Consequently, IL-7R expression by T cells in this strain is almost entirely NF-κB dependent (Silva et al., 2014; **Schematic 5.1**).

TNF induced upregulation of IL-7R expression has previously been shown in F5 thymocytes (Silva et al., 2014). We asked whether the level of IL-7R expression was dose dependent with respect to TNF added *in vitro*. Single cell suspensions of whole, mashed thymii from F5 *Rag1*$^{-/-}$ mice were cultured for 24hr in the presence of increasing concentrations of TNF cytokine or PBS vehicle. As the TNF concentration increased from 0 to 10ng/mL, so did expression of the IL-7R on the CD8SP HSA^{hi} and CD8SP HSA^{lo} subsets of F5 SP thymocytes. Between 10ng/mL and 30ng/mL, IL-7R expression remained constant (**Figure 5.5A**).

CD27 ligand (CD27L, CD70, TNFSF7) is a member of the TNFSF. Like TNF, CD27L has been shown to induce IL-7R expression on the CD8SP thymocytes of F5 *Rag1*$^{-/-}$ mice (Silva et al., 2014). We chose to examine the effects of increasing concentrations of CD27L on IL-7R expression. F5 thymocytes were cultured with soluble CD27L at concentrations ranging from 0 to 100ng/mL. As the concentration of CD27L increased, so did the level of IL-7R expression on the CD8SP HSA^{hi} and CD8SP HSA^{lo} cells (**Figure 5.5B**).

As expected, addition of the highest concentration of TNF (30ng/mL) caused significant death among the CD8SP HSA^{hi} cells. Analysing viability in cultures stimulated with CD27L (100ng/mL) revealed a slight, but not significant, induction of cell death (**Figure 5.5C, left**). Furthermore, comparison of IL-7R expression revealed that CD27L was not as strong an inducer of IL-7R as TNF (**Figure 5.5C, right**).

5.2.7 Regulation of cell death by TNFRSF ligands in WT mice

There are currently 29 recognised members of the TNFRSF (Aggarwal et al., 2012). CD8SP thymocytes of F5 *Rag1*$^{-/-}$ mice have previously been shown to express the following TNFRSF members: TNFR1, TNFR2, TACI, LIGHTR, GITR, TNFRSF25, TNFRSF26, and CD27 (Silva et al., 2014). We also found expression of these receptors on the SP thymocytes of WT, polyclonal mice (data not shown). For this reason we chose to perform cell culture with the following TNFRSF ligands: TNF (ligand for TNFR1 and TNFR2), APRIL and BAFF (ligands for TACI), GITRL (ligand for GITR), LIGHT (ligand for LIGHTR), TLA1 (ligand for TNFRSF25), and TRAIL (ligand for TNFRSF26).

Thymocytes from WT mice, anti-TNF Ab treated IKKΔT^{CD4} mice, *Tnfrsf1a*$^{-/-}$ mice, and *Tnfrsf1a*$^{-/-}$ IKKΔT^{CD4} mice were cultured in the presence of different TNFSF members, or with PBS or IL-7 and, after 24h, cell viability was assessed by FACS. TNF was the only ligand to induce significant death among the SP thymocytes (**Figure 5.6**). As expected, the IKK deficient SP cells that express TNFR1 were very sensitive to TNF (**Figure 5.6**). IL-7 was able to reduce cell death among IKK sufficient cells, but not cells lacking IKK expression (**Figure 5.6**). Strikingly, amongst WT cells, TNF was the only TNFSF member to induce significant upregulation of the IL-7R among SP thymocytes (**Figure 5.7**). Cells deficient in TNFR1, or IKK, or both did not change their IL-7R expression in response to TNF (**Figure 5.7**). Although CD27L promoted IL-7R expression by F5 thymocytes (Silva et al., 2014; **Figure 5.5B & C**), we failed to detect any noticeable IL-7R upregulation among WT thymocytes (**Figure 5.7**). Culture with

IL-7 caused IL-7R downregulation among all strains of SP thymocytes (**Figure 5.7**), as expected (Park et al., 2004).

5.2.8 *In vivo* blockade of CD27 signalling fails to rescue SP thymocyte populations in IKKΔT^{CD4} mice

Culture experiments using F5 thymocytes had suggested that TNF and CD27L might have similar functions in the thymus. Both ligands caused strong upregulation of the IL-7R on F5 SP thymocytes (Silva et al., 2014; **Figure 5.5**). Furthermore, there was some evidience to suggest that CD27L, like TNF, may have a weak cytotoxicity towards CD8SP HSA^{hi} F5 thymocytes (**Figure 5.5C**). We therefore wished to determine whether blocking CD27 signalling in IKKΔT^{CD4} mice could rescue SP populations. IKKΔT^{CD4} mice were treated over a one-week period with anti-CD27L blocking Ab, injected intraperitoneally on days 0, 2, and 4, and numbers of thymic subsets were assessed on day 7. Analysing cell counts at day 7 revealed that treatment of IKKΔT^{CD4} mice with anti-CD27L Ab had no detectable effect on those subsets reduced in this strain. Numbers of CD4SP HSA^{lo}, CD8SP HSA^{hi}, and CD8SP HSA^{lo} thymocytes were not altered by the treatment (**Figure 5.8**). Furthermore, analyzing numbers of naïve cells in the periphery of the IKKΔT^{CD4} mice revealed no increase upon anti-CD27L Ab treatment (**Figure 5.8**).

5.3 Discussion

The aim of this chapter was to investigate factors regulating NF-κB dependent cell survival and IL-7R expression *in vivo*. We, and others, have identified TNF as a ligand that works in an NF-κB dependent manner to upregulate IL-7R expression in SP thymocytes (Silva et al., 2014; **Schematic 5.2A**). In the absence of IKK mediated NF-κB signalling, TNF induces substantial death among the SP population and no increase in IL-7R.

We used a combined genetic and pharmacological approach to acutely block NF-κB signalling in thymocytes. IKK1 deficient SP cells are no more sensitive to TNF induced death than their WT counterparts (**Figure 5.3A**). However, *in vitro* addition of an IKK2 inhibitor to the IKK1 deficient thymocytes resulted in much TNF induced cell death among all SP subsets (**Figure 5.3**; **Schematic 5.2B**). Importantly, this indicates that an acute block in NF-κB signalling is sufficient to render SP cells susceptible to TNF. We had hypothesized that NF-κB may be required at discrete developmental stages, in order to produce a TNF resistant population of cells. However, from our results, it seems that NF-κB plays a very active, as opposed to developmental, role in protecting SP thymocytes from TNF induced death.

In vitro experiments confirmed that, when NF-κB signalling is blocked, thymocyte susceptibility to TNF induced death increases in the order: CD4SP HSA^{hi}, CD4SP HSA^{lo}, CD8SP HSA^{hi}, CD8SP HSA^{lo} (**Figure 5.3**). This is reflected in the phenotype of the intact $IKK\Delta T^{CD4}$ mice. In the intact animals, CD8SP thymocytes are decreased relative to CD4SP thymocytes, and the HSA^{lo} subsets are more affected than the HSA^{hi} subsets. Through an *in vitro* approach we were able to remove anatomical barriers. Hence we can be confident that all SP thymocyte subsets investigated were subjected to the same concentration of TNF. Importantly, culture experiments have shown that differences in TNF susceptibility among the SP populations are cell intrinsic (**Figure 5.1**; **Figure 5.2**; **Figure 5.3**). Nevertheless, it is likely that thymocytes are subjected to differing concentrations of TNF as they develop.

Maturing thymocytes are highly motile, experiencing many different thymic microenvironments as they progress from their least to most mature stages. DN thymocytes mature through DN1-4, moving from the cortico-medullary junction, through the thymic cortex and towards the subcapsular zone as they do so (Takahama, 2006). DP cells are then generated in the cortex of the thymus where they interact with peptide:MHC complexes on cortical thymic epithelial cells and dendritic cells. A tiny percentage (3-5%) of DP cells undergo positive selection and migrate from the cortex to the medulla (Takahama, 2006). DP thymocytes may be found in both the outer and inner areas of the cortex, depending on whether they are in the early or late stages of positive selection (Germain, 2002). Newly generated SP thymocytes reside in the medulla for approximately 4 days, undergoing the final stages of maturation, before leaving the thymus through the perivascular space (Sinclair et al., 2011; Takahama, 2006). Reportedly, medullary epithelial cells are constitutive producers of TNF (Mahmud et al., 2014). Therefore, it is likely that all SP thymocytes experience much TNF induced signalling during their transition from the HSA^{hi} to HSA^{lo} stage.

In pLck-IκB-PEST mice, only the CD8SP HSA^{lo} thymocyte population is affected by TNF induced death. Via cell culture, we found that 0.1ng/mL TNF was enough to induce significant death among CD8SP HSA^{lo} cells from pLck-IκB-PEST mice, but that all other subsets required at least 1ng/mL before significant death was seen (**Figure 5.2**). This suggests that the physiological concentration of TNF in the thymus is likely around 0.1ng/mL or less and not more than 1ng/mL.

The presence of TNF in the thymic medulla implies that it plays a role in the final stages of thymocyte maturation. Indeed TNFR2 is upregulated on T_{reg} cells during their thymic development (Mahmud et al., 2014). Furthermore, we showed that the addition of TNF *in vitro* causes significant upregulation of the IL-7R on WT, SP thymocytes (**Figure 5.4**; **Figure 5.7**). In addition to its presumed role in T_{reg} production, an important role for TNF in the thymus may well be to modulate IL-7R expression. During thymocyte development, IL-7R is expressed during the DN stage, lost during the DP stage, and re-expressed

after positive selection (Sinclair et al., 2011; Sudo et al., 1993). Evidence suggests that the strength of TCR signalling during positive selection is responsible for the amount of IL-7R re-expression (Sinclair et al., 2011). For this reason, F5 thymocytes, which have only weak positive selection signalling via their transgenic TCR, normally lack expression of IL-7R during the SP stage (Sinclair et al., 2011; **Schematic 5.1**). However, when TNF was added to a culture of F5 thymocytes, the SP cells were able to dramatically increase IL-7R expression, in a manner dependent on the concentration of TNF used (**Figure 5.5A**). As T cells leave the thymus, to become recent thymic emigrants (RTEs) in the periphery, they will continue to upregulate their IL-7R expression (Silva et al., 2014).

Runx1 and Runx3 transcription factors are believed to be important for the re-expression of the IL-7R on CD4 and CD8 SP cells after positive selection (Egawa et al., 2007). However, the further upregulation of the IL-7R, as cells leave the thymus to become RTEs, is believed to be highly NF-κB dependent (Silva et al., 2014). If T cells experience a lack of NF-κB signalling during thymic development, they will exhibit reduced lL-7R expression when in their naïve, peripheral form (Miller et al., 2014; Silva et al., 2014). Importantly, NF-κB signalling does not appear to be required to maintain IL-7R levels on mature, naïve cells (Silva et al., 2014), although it may be required to restore IL-7R levels following downregulation induced by *in vitro* addition of IL-7 cytokine (Miller et al., 2014).

In IKK2 deficient mice, IL-7R expression is normal among HSA^{hi} SP thymocytes and CD4SP HSA^{lo} thymocytes, but significantly reduced among CD8SP HSA^{lo} cells (Silva et al., 2014). We also found this to be true for $IKK\Delta T^{CD4}$ mice (data not shown). However, it seemed that all SP subsets in $IKK\Delta T^{CD4}$ mice showed some degree of functional deficiency in IL-7R signalling. Whilst, *in vitro* addition of IL-7 cytokine significantly reduced death among NF-κB sufficient SP thymocytes, it had limited capacity to promote survival of thymocytes from IKK deficient mice (**Figure 5.6**).

In vitro addition of TNF increases the IL-7R levels on SP cells (Silva et al., 2014; **Figure 5.4**; **Figure 5.5A & C**; **Figure 5.7**). However, if the cells are deficient in IKK2, then the extent of upregulation is limited (Silva et al., 2014), and if the cells are deficient in both IKK1 and IKK2, then there is no TNF induced IL-7R upregulation (**Figure 5.4A, bottom**; **Figure 5.7**). Only limiting amounts of IL-7 cytokine are available in the thymic microenvironment, so regulation of IL-7R expression is particularly important in the thymus (Park et al., 2004). TNF may help provide such regulation. It seems that TNF signalling may be a useful activator of NF-κB in SP thymocytes - enabling upregulation of the IL-7R in the later stages of thymocyte development, just before cells enter the periphery as RTEs. Notably, thymocytes that do not upregulate IL-7R sufficiently fail to survive in the periphery (Sinclair et al., 2011). Hence, TNF induced IL-7R upregulation may ultimately help to shape the T cell repertoire.

As discussed in **Chapter 4**, T cells from *Tnfrsf1a*$^{-/-}$ mice have completely normal IL-7R expression. It is possible that TNF signalling could still occur in TNFR1 deficient cells via TNFR2. However, anti-TNF Ab treated WT mice also had normal IL-7R expression. These results suggest that TNF signalling is not essential for the induction of IL-7R expression in the last stages of T cell development (**Schematic 5.2A**). Of note, there is much redundancy among members of the TNFSF – all of which can activate NF-κB signalling. F5 thymocytes express TNFR1, TNFR2, TACI, LIGHTR, GITR, TNFRSF25, TNFRSF26, and CD27 (Silva et al., 2014). Like TNF, CD27L has been shown to induce IL-7R expression on the SP thymocytes of F5 *Rag1*$^{-/-}$ mice (Silva et al., 2014; **Figure 5.5B & C**). However, when WT, polyclonal thymocytes were cultured with CD27L there was no obvious IL-7R upregulation (**Figure 5.7**). Similarly, culture with other TNFSF members failed to show any dramatic effect (Silva et al., 2014; **Figure 5.7**). However, if NF-κB activation is receptor dependent in developing thymocytes, it appears there is redundancy between multiple receptors to perform this function (**Schematic 5.2A**).

Evidence suggests that TNF and CD27L may have similar biological functions in the thymus (Coquet et al., 2013; Mahmud et al., 2014; Silva et al., 2014; **Figure 5.5**). However, unlike TNF, CD27L could not induce death among IKK

deficient cells *in vitro* (**Figure 5.6**). Furthermore, treatment with anti-CD27L blocking Ab failed to increase cell numbers in IKKΔT^{CD4} mice (**Figure 5.8**). CD27 does not contain an intracellular death domain. However, CD27 is able to induce cell death via interaction with the death domain containing protein, Siva (Prasad et al., 1997). Hence, CD27L induced death, although not observed, remains a possibility among thymocytes. Whether TNFSF member (e.g. TNF or CD27L) induced apoptosis has any physiological relevance in the WT thymus remains to be determined.

In this chapter we discovered that, in the absence of NF-κB signalling, certain SP thymocyte populations are intrinsically more susceptible to TNF induced death than others. Importantly, we found that NF-κB works to actively protect SP cells from TNF. Furthermore, we have speculated that the TNFSF members TNF and CD27L may play a functional role in the thymus by contributing to the NF-κB dependent upregulation of the IL-7R during the final stages of thymocyte maturation. In **Chapter 6**, we shall further investigate the mechanism behind the TNF induced death of SP thymocytes. Ultimately, we hope to determine why certain SP subsets show greater susceptibility than others to TNF induced cell death in the absence of NF-κB signalling.

Figure 5.1 Upon *in vitro* addition of TNF, mature thymocytes from IKKΔT^{CD4} mice appear highly susceptible to death

(**A**) Single cell suspensions of whole, mashed thymii from indicated mice were cultured for a period of 24 hours. Prior to this, "IKKΔT^{CD4} αTNF treated" mice were treated with an anti-TNF blocking antibody over a period of 7 days, as were "WT αTNF treated" mice. "IKKΔT^{CD4}" mice and "WT" mice either received no treatment or received PBS vehicle for 7 days prior to culture (PBS injections were found to have no measurable effect). "*Tnfrsf1a*$^{-/-}$" mice and "*Tnfrsf1a*$^{-/-}$ IKKΔT^{CD4}" mice received no treatment prior to the culture experiments. Throughout the culture period, culture medium contained PBS vehicle, TNF cytokine (30ng/mL), or IL-7 cytokine (10ng/mL), as indicated. The percentage of dead cells within each thymocyte subset is shown. Bar charts display data collected from a number of independent experiments. (**B**) DP1 (TCR^{lo}CD5^{lo}), DP2 (TCR^{int}CD5^{hi}), and DP3 (TCR^{hi}CD5^{int}) populations were sorted from WT thymii. The sorted cells were then cultured for 24hr with medium containing either PBS vehicle or TNF cytokine. Density plots show sorted populations before culture (0hr) and after culture (24hr). Bar charts display the percentage cell death among the DP subsets. Data collected from 2 independent cell sorts and culture experiments are shown.

Bar charts show mean ± SEM. ns = not significant; * = significant at P<0.05; ** = significant at P<0.01; *** = significant at P<0.001.

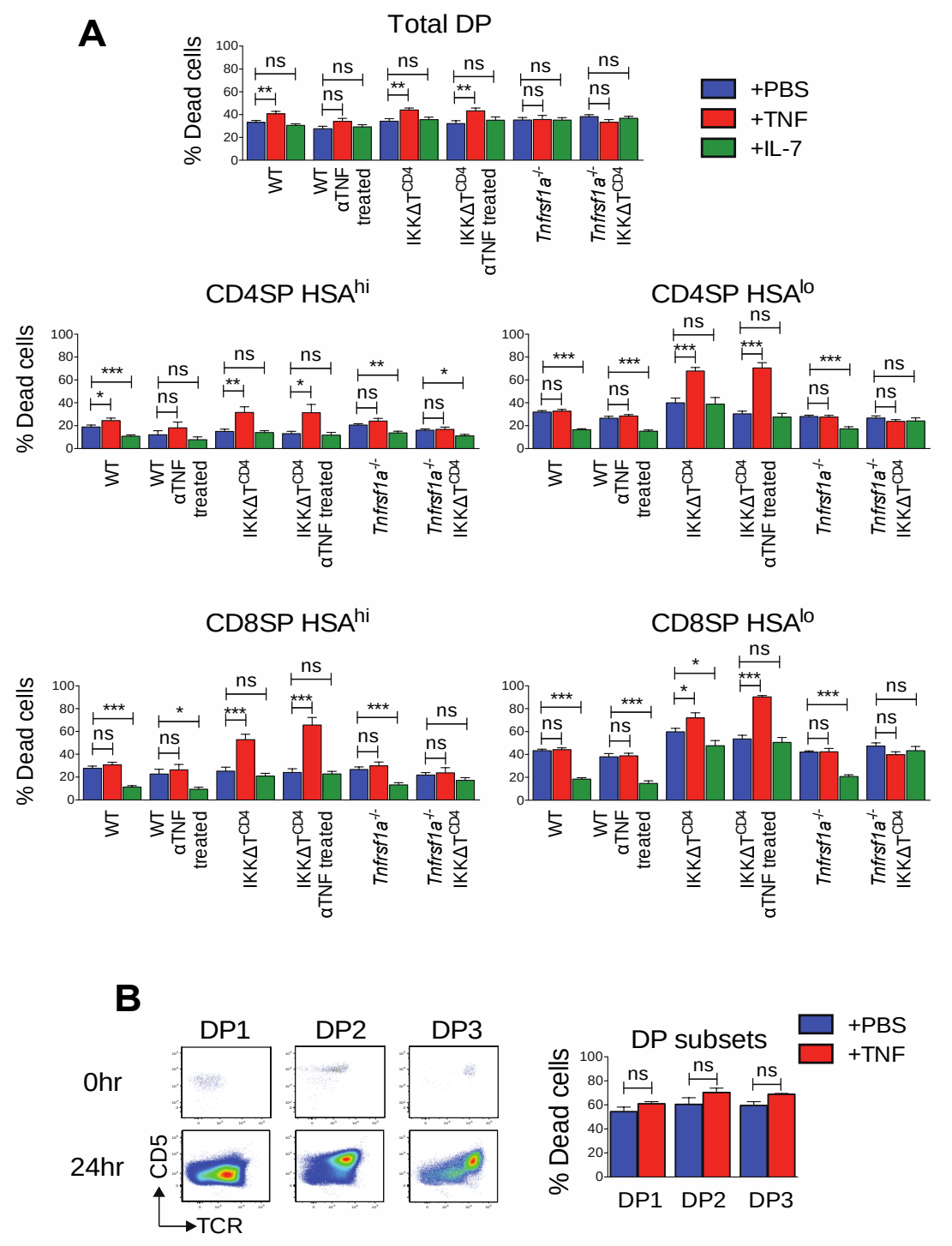

A
Total DP
% Dead cells
+PBS
+TNF
+IL-7
WT
WT αTNF treated
IKKΔT^CD4
IKKΔT^CD4 αTNF treated
Tnfrsf1a^-/-
Tnfrsf1a^-/- IKKΔT^CD4
CD4SP HSA^hi
CD4SP HSA^lo
CD8SP HSA^hi
CD8SP HSA^lo
B
DP1
DP2
DP3
0hr
24hr
CD5
TCR
DP subsets

Figure 5.2 When NF-κB signalling is blocked, via expression of a dominant negative form of IκBα, CD8SP HSA^{lo} cells are particularly sensitive to TNF induced death

The thymus was taken from a WT mouse and from a pLck-IκB-PEST mouse (expressing a super-inhibitory form of IκBα under the control of the proximal Lck promoter). Single cell suspensions were made and the thymocytes cultured for a 24hr period with increasing concentrations of TNF cytokine. The percentage of dead cells within each SP thymocyte population is shown. Error bars show mean ± SEM. The Student's t-test was performed on pLck-IκB-PEST thymocytes. ns = not significant; * = significant at $P<0.05$; ** = significant at $P<0.01$. The experiment was performed once. Duplicate wells were employed.

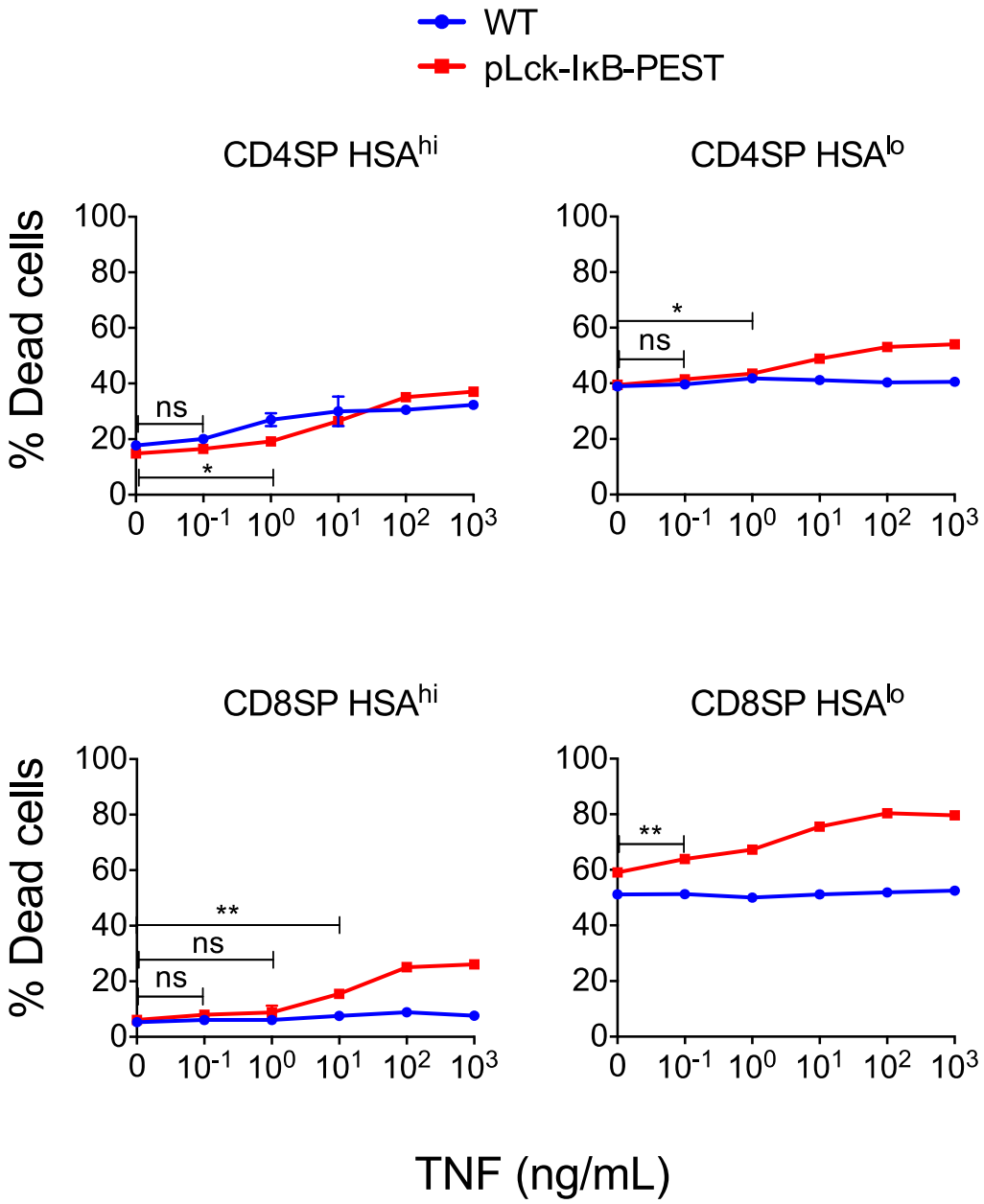
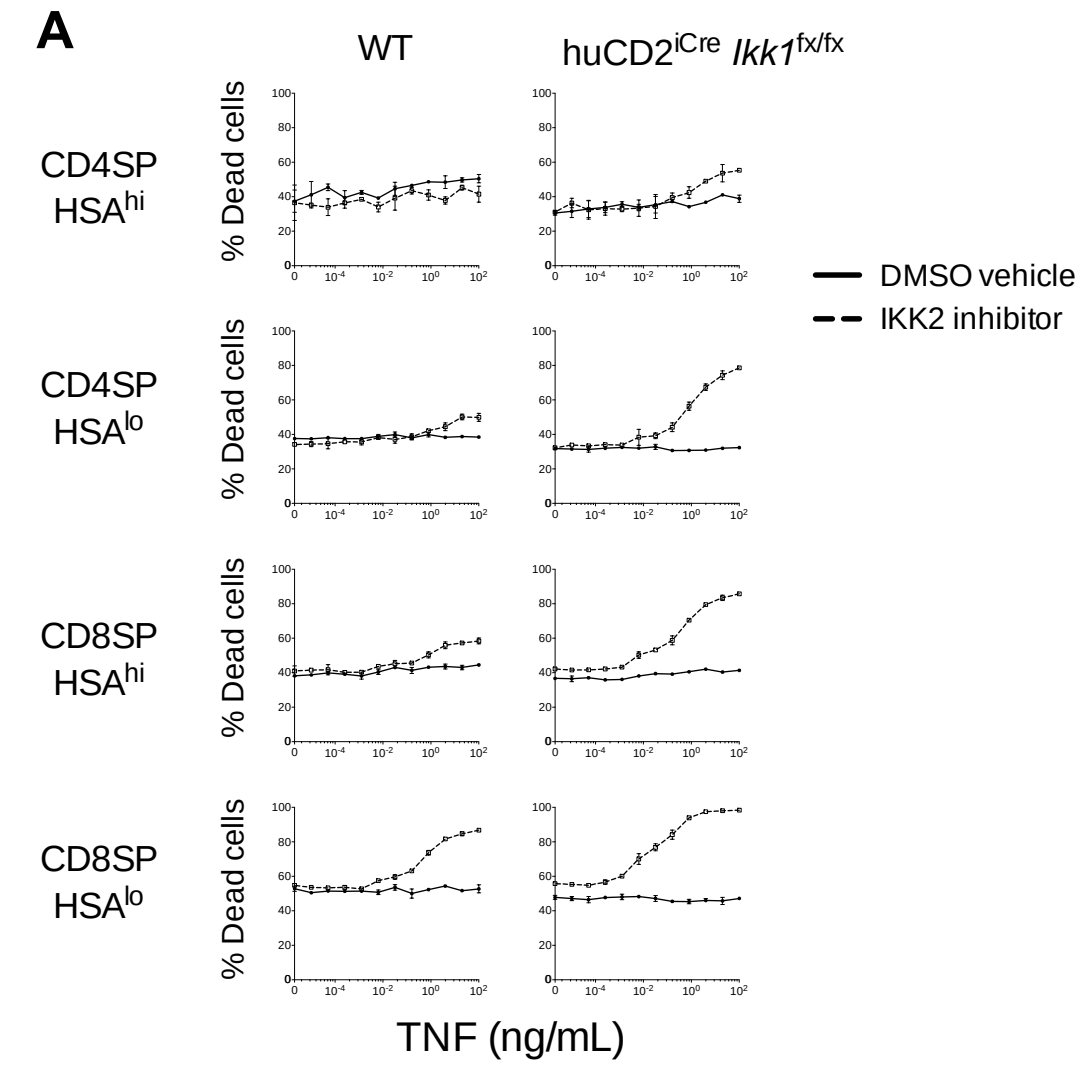

WT
pLck-IκB-PEST
CD4SP HSA^hi
CD4SP HSA^lo
CD8SP HSA^hi
CD8SP HSA^lo
% Dead cells
% Dead cells
ns
*
ns
*
ns
ns
**
**
0
20
40
60
80
100
0
10^-1
10^0
10^1
10^2
10^3
TNF (ng/mL)

Figure 5.3 When NF-κB signalling is deficient, certain SP thymocyte populations show a greater sensitivity to TNF induced death than others

(**A**) Thymii from 3 huCD2^{iCre} *Ikk1*$^{fx/fx}$ R26R^{EYFP} mice were pooled together, as were thymii from 3 Cre- (WT) littermates. Single cell suspensions were made. Either the IKK2 specific inhibitor BI605906 (10μM) or DMSO vehicle was added to the huCD2^{iCre} *Ikk1*$^{fx/fx}$ R26R^{EYFP} cells or the WT cells. TNF was also present in the culture medium at varying concentrations. Cells were cultured for 24 hours. The percentage of dead cells is shown for each SP thymocyte population. Technical replicates were employed by way of duplicate wells. The experiment was performed twice. One representative experiment is shown. (**B**) Nonlinear regression was performed on the data collected from the huCD2^{iCre} *Ikk1*$^{fx/fx}$ R26R^{EYFP} cells treated with IKK2 inhibitor. Dotted lines represent the IC_{50} values for the different SP subsets. IC_{50} values are as follows: CD4SP HSA^{hi}, 1.3ng/mL; CD4SP HSA^{lo}, 1ng/mL; CD8SP HSA^{hi}, 0.3ng/mL; CD8SP HSA^{lo}, 0.03ng/mL. Wild-type cells (treated with DMSO vehicle) are directly compared to the huCD2^{iCre} *Ikk1*$^{fx/fx}$ R26R^{EYFP} cells treated with IKK2 inhibitor. The experiment was performed twice. One representative experiment is shown.

Line graphs show mean ± SEM.


A
WT
huCD2iCre Ikk1fx/fx
CD4SP HSAhi
CD4SP HSAlo
CD8SP HSAhi
CD8SP HSAlo
% Dead cells
DMSO vehicle
IKK2 inhibitor
TNF (ng/mL)

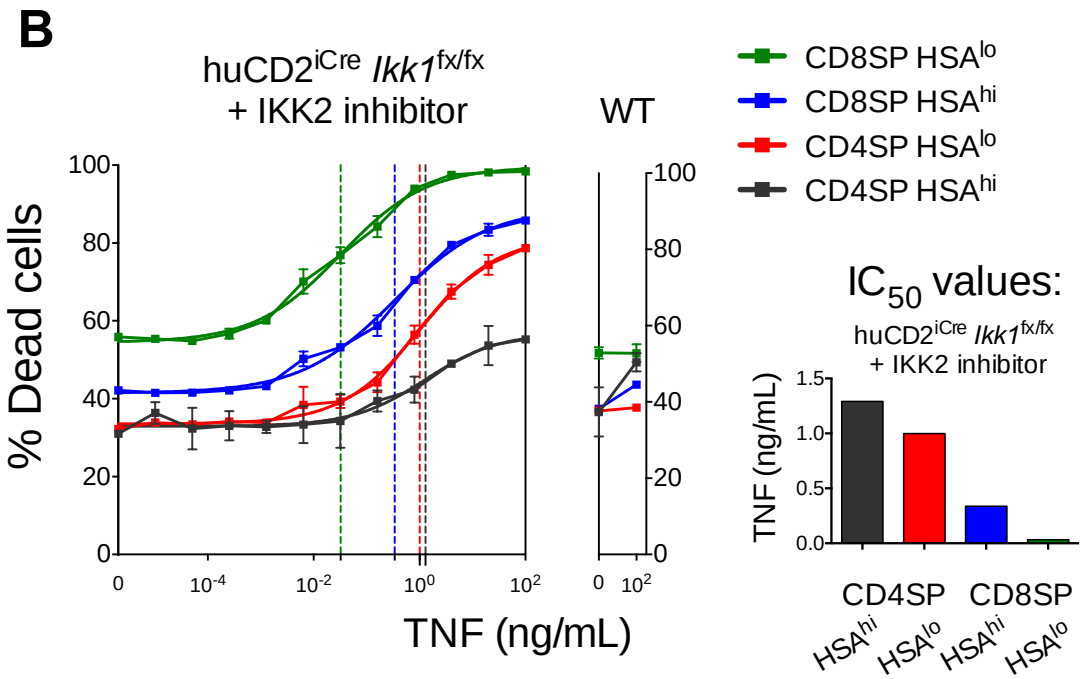
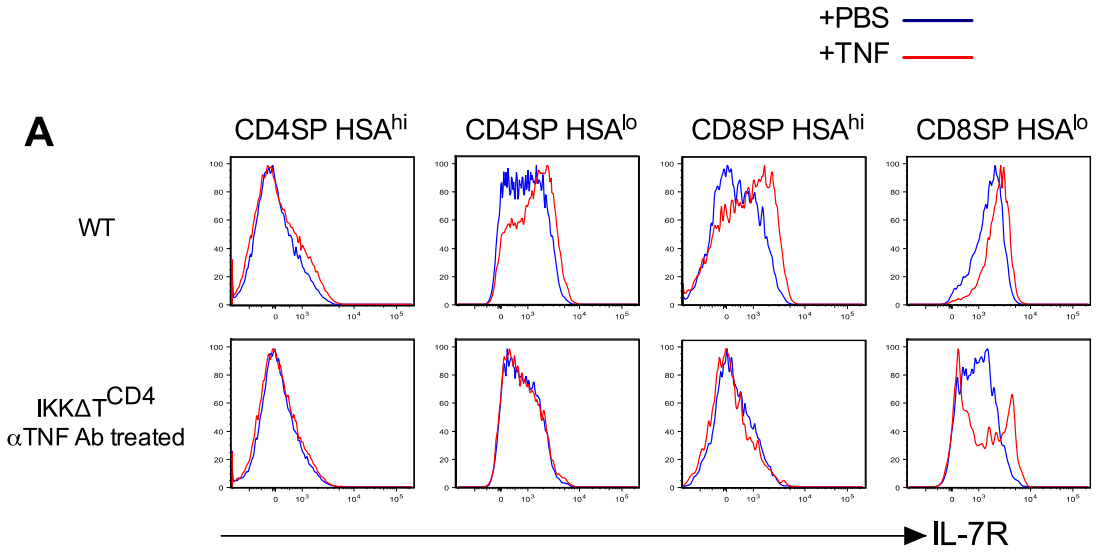
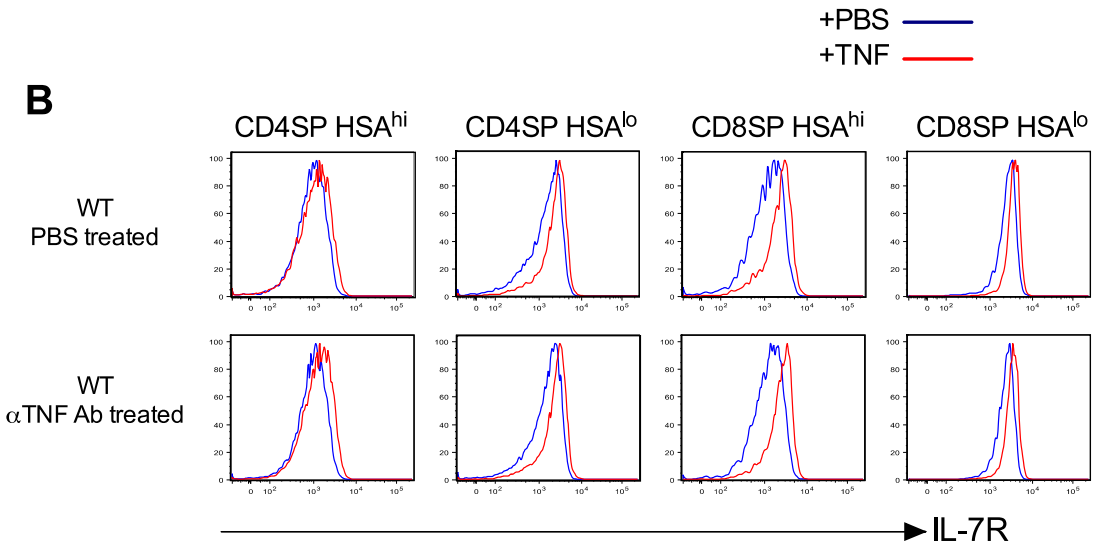

B
huCD2iCre Ikk1fx/fx
+ IKK2 inhibitor
WT
CD8SP HSAlo
CD8SP HSAhi
CD4SP HSAlo
CD4SP HSAhi
% Dead cells
TNF (ng/mL)
IC50 values:
huCD2iCre Ikk1fx/fx
+ IKK2 inhibitor
TNF (ng/mL)
CD4SP
CD8SP
HSAhi
HSAlo
HSAhi
HSAlo

Figure 5.4 Upon *in vitro* addition of TNF, mature thymocytes from IKKΔT^{CD4} mice lack induced expression of the IL-7R

(**A**) IKKΔT^{CD4} mice were treated over a 7 day period with an anti-TNF blocking antibody. This increased the number of IKK1/2 double deficient SP thymocytes and also increased the likelihood that the cells present were truly IKK deficient (and not "*escapants*"). Single cell suspensions of whole, mashed thymii from these treated mice or from untreated Cre- (WT) littermates were then cultured for a 24hr period in medium containing either PBS vehicle or TNF cytokine (30ng/mL). SP thymocytes were then analysed for expression of the IL-7R. The experiment was repeated a number of times, results shown are representative. (**B**) C57BL/6 (WT) mice were treated over a 7 day period with an anti-TNF blocking antibody or PBS vehicle. Single cell suspensions of whole, mashed thymii from these mice were then cultured for a 24hr period in medium containing either PBS vehicle or TNF cytokine (30ng/mL). SP thymocytes were then analysed for expression of the IL-7R. The experiment was repeated twice, results shown are representative.


+PBS
+TNF
A
CD4SP HSAhi
CD4SP HSAlo
CD8SP HSAhi
CD8SP HSAlo
WT
IKKΔTCD4
αTNF Ab treated
IL-7R


+PBS
+TNF
B
CD4SP HSAhi
CD4SP HSAlo
CD8SP HSAhi
CD8SP HSAlo
WT
PBS treated
WT
αTNF Ab treated
IL-7R

Schematic 5.1 Upregulation of the IL-7R during thymocyte development

The survival of DN thymocytes relies on signalling through the IL-7R (Peschon et al., 1994). By the DP stage, however, thymocytes completely lack cell surface expression of the IL-7R (Sudo et al., 1993). During the process of positive selection, signalling via the TCR induces upregulation of the IL-7R (Sinclair et al., 2011). Hence, by the SP stage, both CD4 and CD8 lineage cells show high IL-7R expression. However, in F5 *Rag1*$^{-/-}$ mice, suboptimal signalling via the transgenic TCR means that SP thymocytes continue to lack IL-7R expression (Sinclair et al., 2011). As SP thymocytes undergo the final stages of maturation and begin to leave the thymus as recent thymic emigrants (RTEs) they further increase their IL-7R expression in a manner dependent on activation of the NF-κB transcription factor (Silva et al., 2014). IL-7R expression by the T cells of F5 *Rag1*$^{-/-}$ mice is almost entirely NF-κB dependent (Silva et al., 2014). In contrast, SP thymocytes deficient in NF-κB signalling will fail to upregulate their IL-7R as they exit the thymus, resulting in low IL-7R expression among peripheral T cells (Silva et al., 2014). As explored in **Chapter 3**, the greater the block in NF-κB signalling during T cell development, the lower the level of IL-7R expression among the naïve T cell population. Maintenance of the IL-7R on mature, peripheral T cells is dependent on the Foxo1 transcription factor (Kerdiles et al., 2009) and seems independent of NF-κB (Silva et al., 2014).

Figure drawn by myself, but based on similar diagrams as presented by Benedict Seddon.

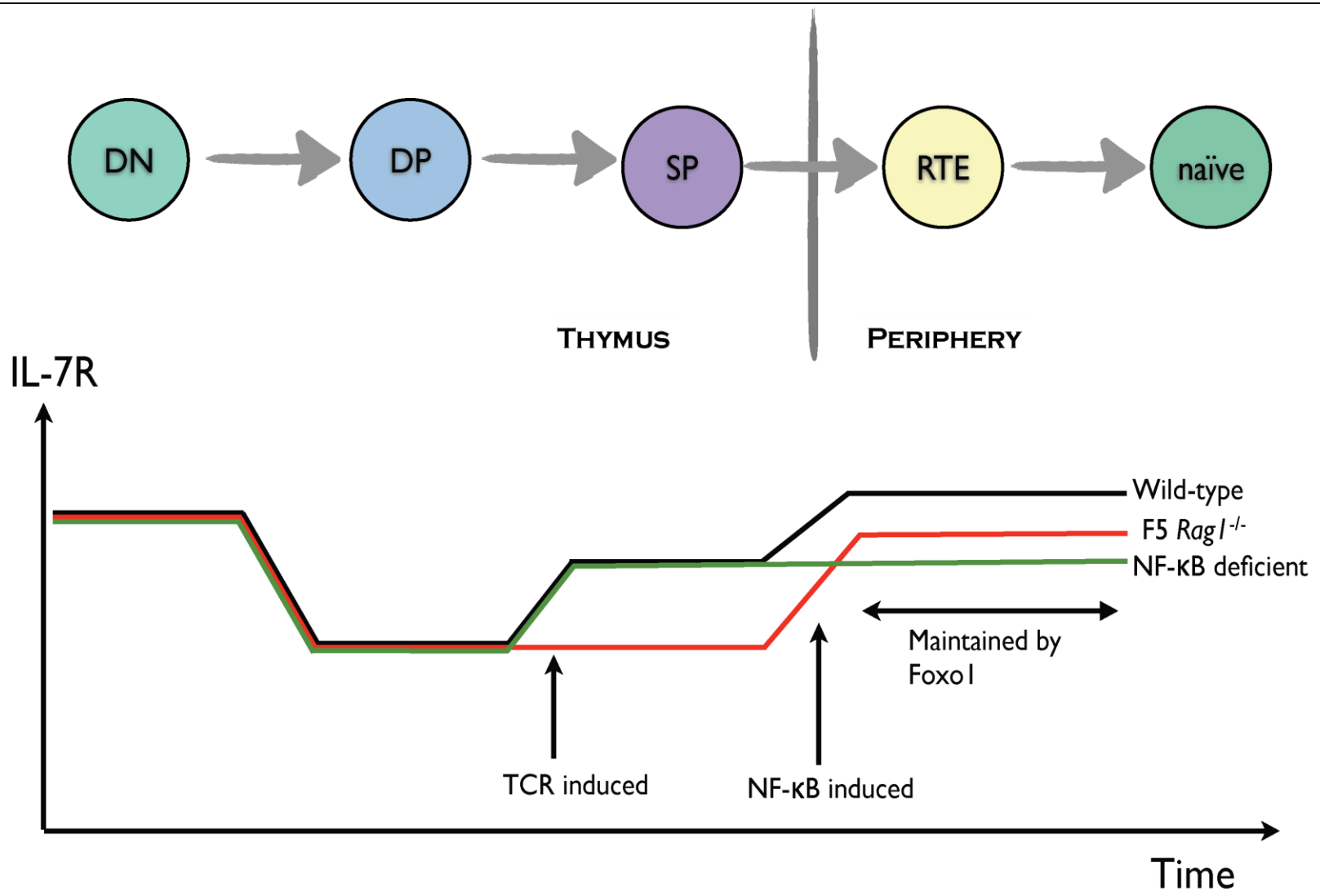
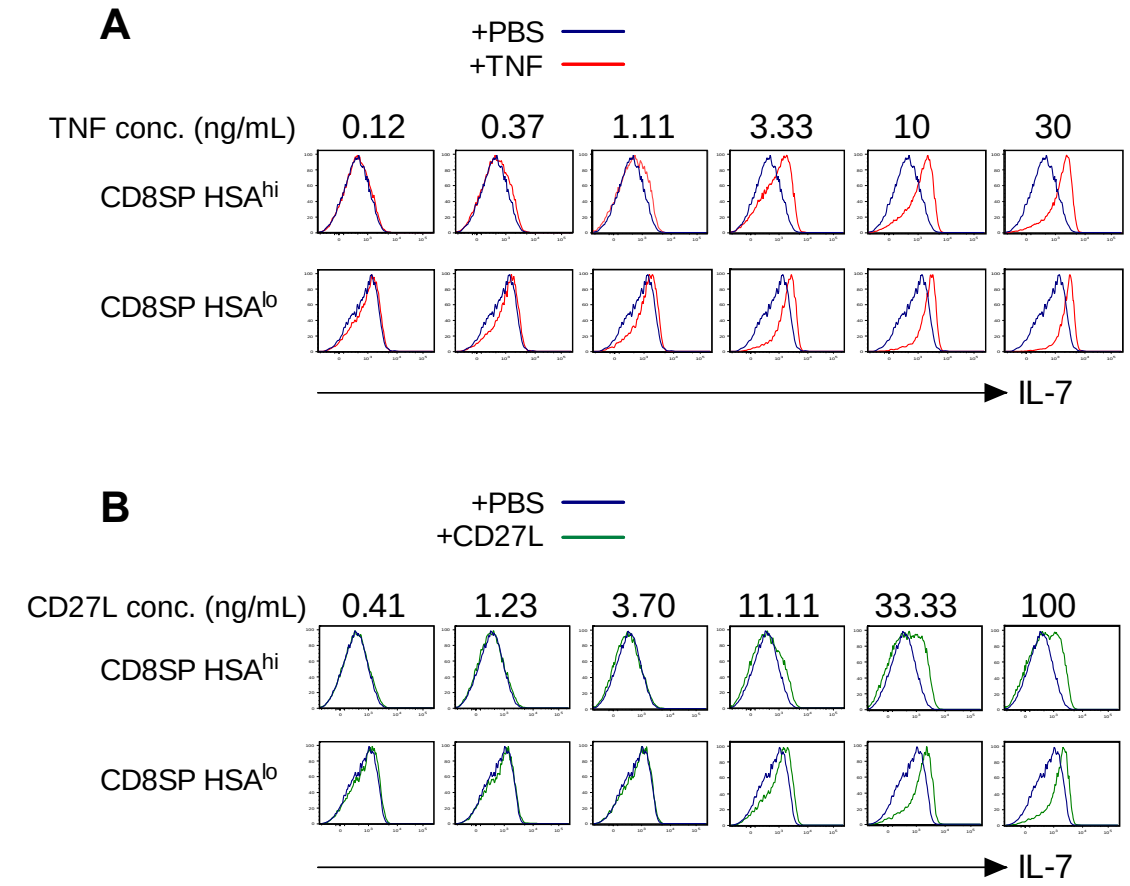

DN
DP
SP
RTE
naïve
THYMUS
PERIPHERY
IL-7R
Wild-type
F5 Rag1-/-
NF-κB deficient
Maintained by
Foxo1
TCR induced
NF-κB induced
Time

Figure 5.5 *In vitro* culture with TNF or with CD27L causes upregulation of the IL-7R on the CD8SP thymocytes of F5 *Rag1*$^{-/-}$ mice

Single cell suspensions of whole, mashed thymii from F5 *Rag1*$^{-/-}$ mice were cultured for a period of 24hr in medium containing varying concentrations of TNF, CD27L, or PBS control. At the end of the experiment, cells were stained and analysed by FACS. (**A**) IL-7R expression is shown for CD8SP HSA^{hi} (top row) and CD8SP HSA^{lo} (bottom row) cells cultured with TNF vs PBS. (**B**) IL-7R expression is shown for CD8SP HSA^{hi} (top row) and CD8SP HSA^{lo} (bottom row) cells cultured with CD27L vs PBS. (**A** & **B**) Data are representative of biological (2 mice) and technical (2 well) replicates. (**C**) Thymocytes from F5 *Rag1*$^{-/-}$ mice were cultured for 24hr in PBS vehicle, 10ng/mL IL-7, 30ng/mL TNF, or 100ng/mL CD27L. Following culture, the CD8SP HSA^{hi} thymocyte population was assessed for viability (left) and for expression of the IL-7R (right). The percentage increase in IL-7R mean fluorescence intensity (MFI) when IL-7, TNF, or CD27L was present, as compared to PBS vehicle, was calculated. The left bar chart shows data from 5 independent experiments. The right bar chart shows data from 3 independent experiments. Bar charts show mean ± SEM. ns = not significant; * = significant at P<0.05; *** = significant at P<0.001.


A
+PBS
+TNF
TNF conc. (ng/mL)
0.12
0.37
1.11
3.33
10
30
CD8SP HSAhi
CD8SP HSAlo
IL-7
B
+PBS
+CD27L
CD27L conc. (ng/mL)
0.41
1.23
3.70
11.11
33.33
100
CD8SP HSAhi
CD8SP HSAlo
IL-7

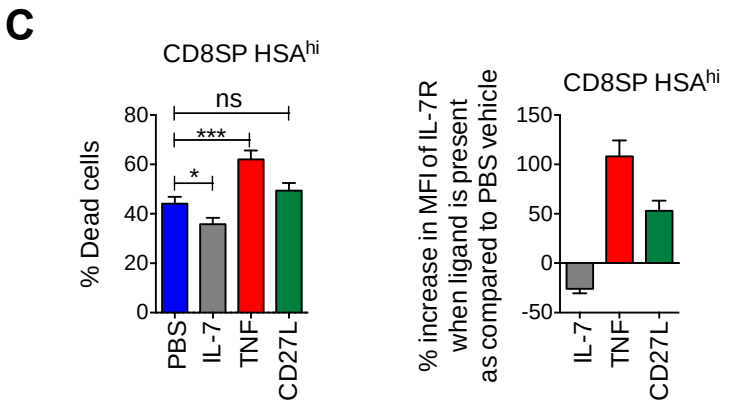

C
CD8SP HSAhi
% Dead cells
ns
***
*
80
60
40
20
0
PBS
IL-7
TNF
CD27L
CD8SP HSAhi
% increase in MFI of IL-7R when ligand is present as compared to PBS vehicle
150
100
50
0
-50
IL-7
TNF
CD27L

Figure 5.6 In contrast to TNF itself, other ligands of the TNF superfamily fail to induce death in IKKΔT^{CD4} cells

Thymocytes from the indicated mouse strains were cultured for 24hr in the presence of medium containing a ligand belonging to the TNF superfamily, IL-7 cytokine, or PBS vehicle. SP thymocyte populations were then gated upon using FACS and cells assessed for viability. Prior to culture, "αTNF treated IKKΔT^{CD4}" mice were treated over a 7 day period with an anti-TNF blocking antibody. "WT" mice were the Cre- littermates of the IKKΔT^{CD4} mice. They either received anti-TNF blocking antibody injections, PBS injections, or no treatment prior to cell culture (the injections having no measurable outcome on WT mice). "*Tnfrsf1a*$^{-/-}$" mice were the Cre- littermates of "*Tnfrsf1a*$^{-/-}$ IKKΔT^{CD4}" mice. *Tnfrsf1a*$^{-/-}$ mice and *Tnfrsf1a*$^{-/-}$ IKKΔT^{CD4} mice were not treated prior to culture. The bar charts show data collected from 6 independent experiments. Bar charts show mean ± SEM of at least 2 mice. ns = not significant; * = significant at P<0.05; ** = significant at P<0.01; *** = significant at P<0.001. IL-7 was used at 10ng/mL, TNF was used at 30ng/mL, all other TNFSF cytokines were used at 100ng/mL.

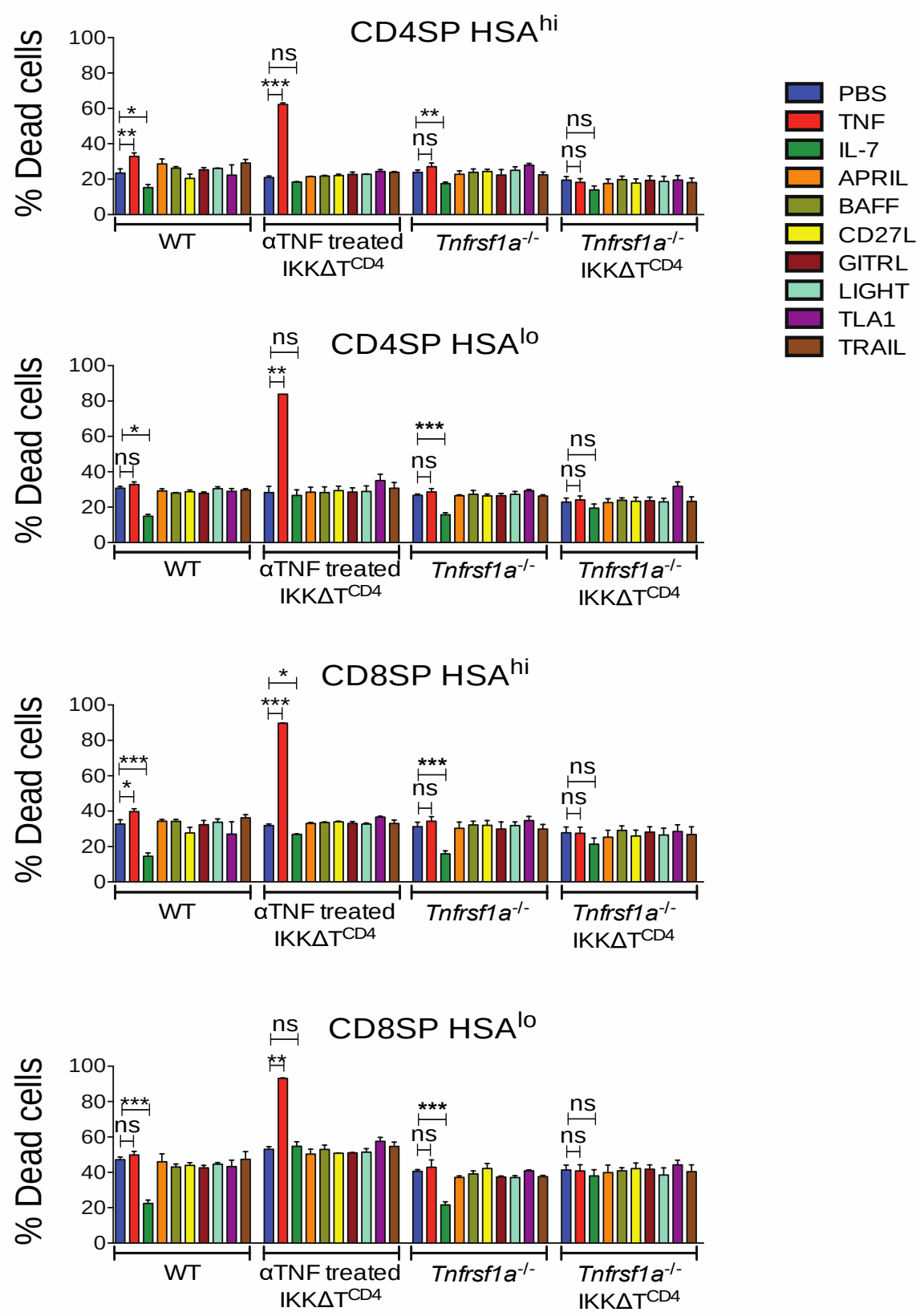

CD4SP HSA^hi
CD4SP HSA^lo
CD8SP HSA^hi
CD8SP HSA^lo
% Dead cells
100
80
60
40
20
0
WT
αTNF treated IKKΔT^CD4
Tnfrsf1a^-/-
Tnfrsf1a^-/- IKKΔT^CD4
PBS
TNF
IL-7
APRIL
BAFF
CD27L
GITRL
LIGHT
TLA1
TRAIL

Figure 5.7 In contrast to TNF itself, other ligands of the TNF superfamily fail to cause upregulation of the IL-7R in NF-κB sufficient, polyclonal mice

Thymocytes from the indicated mouse strains were cultured for 24hr in the presence of medium containing a ligand belonging to the TNF superfamily, IL-7 cytokine, or PBS vehicle. SP thymocyte populations were then gated upon using FACS, and live cells were further assessed for their expression of the IL-7R. The percentage increase in IL-7R mean fluorescence intensity (MFI) when the ligand of interest was present, as compared to PBS vehicle, could then be calculated. Prior to culture, "αTNF treated IKKΔT^{CD4}" mice were treated over a 7 day period with an anti-TNF blocking antibody. "WT" mice were the Cre- littermates of the IKKΔT^{CD4} mice. They either received anti-TNF blocking antibody injections, PBS injections, or no treatment prior to cell culture (the injections having no measurable outcome on WT mice). "*Tnfrsf1a*$^{-/-}$" mice were the Cre- littermates of "*Tnfrsf1a*$^{-/-}$ IKKΔT^{CD4}" mice. *Tnfrsf1a*$^{-/-}$ mice and *Tnfrsf1a*$^{-/-}$ IKKΔT^{CD4} mice were not treated prior to culture. The bar charts show data collected from 7 independent experiments. Bar charts show mean ± SEM of at least 2 mice. IL-7 was used at 10ng/mL, TNF was used at 30ng/mL, all other TNFSF cytokines were used at 100ng/mL.

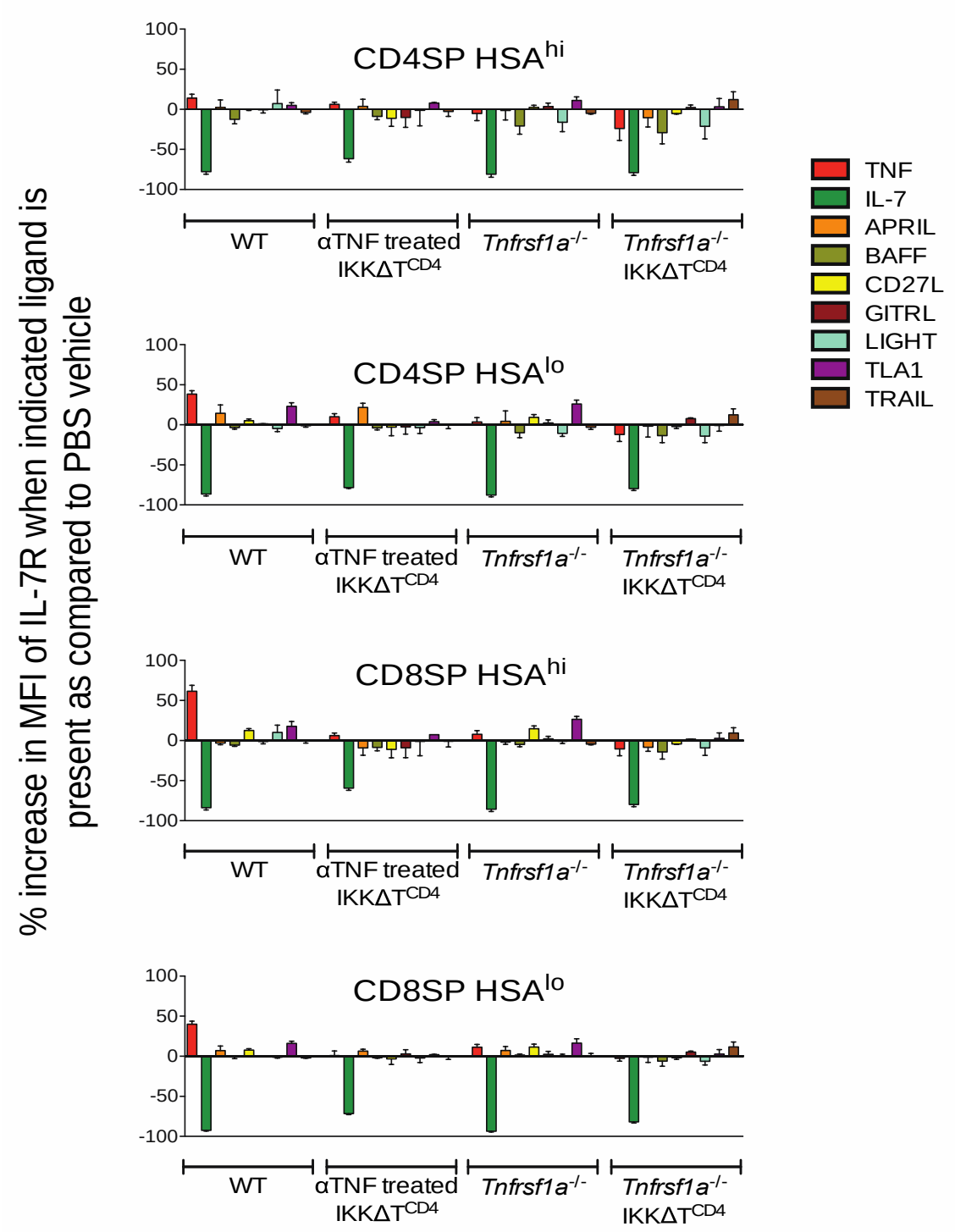
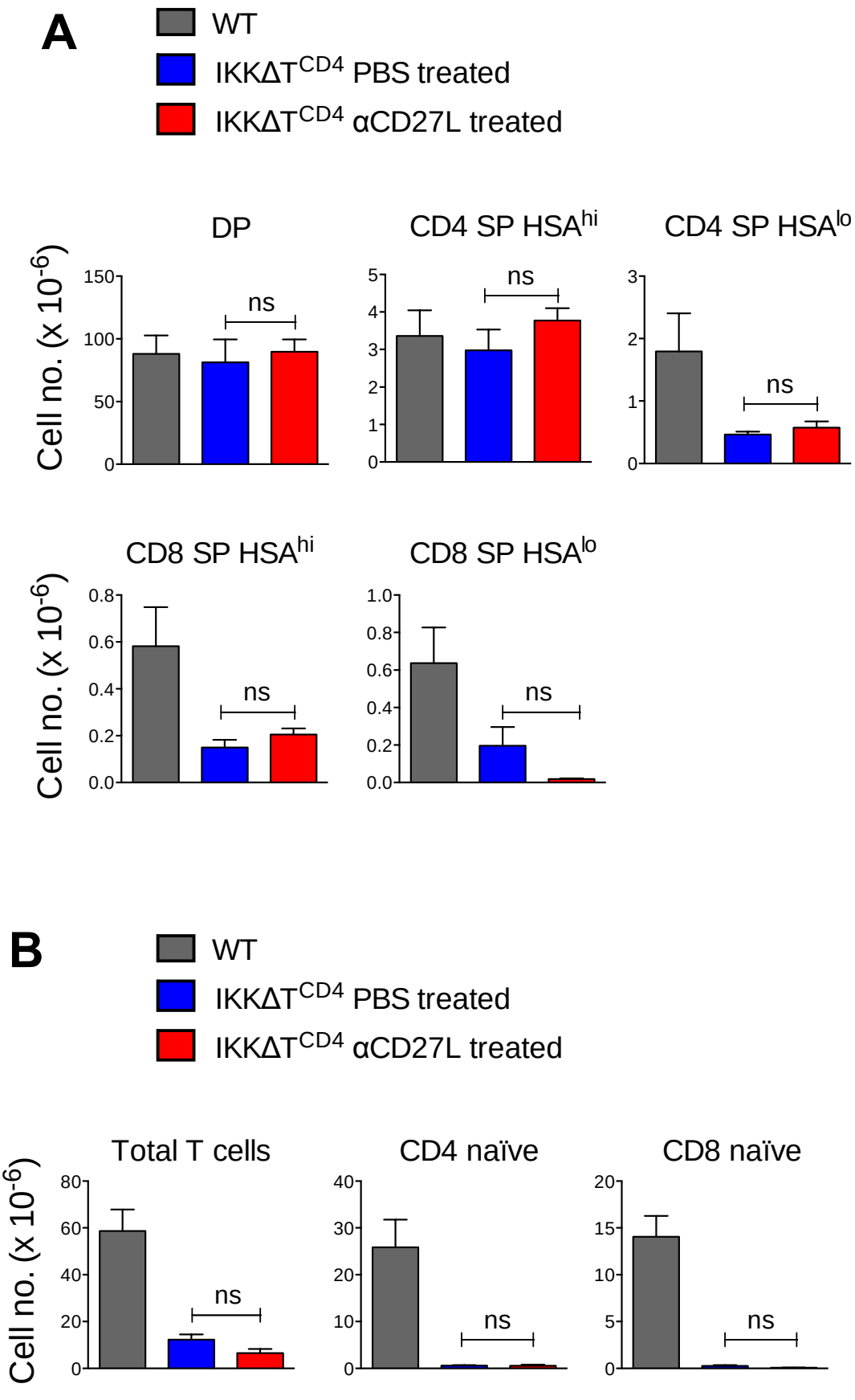

% increase in MFI of IL-7R when indicated ligand is present as compared to PBS vehicle
CD4SP HSA^hi
CD4SP HSA^lo
CD8SP HSA^hi
CD8SP HSA^lo
100
50
0
-50
-100
WT
αTNF treated IKKΔT^CD4
Tnfrsf1a^-/-
Tnfrsf1a^-/- IKKΔT^CD4
TNF
IL-7
APRIL
BAFF
CD27L
GITRL
LIGHT
TLA1
TRAIL

Figure 5.8 Treatment of IKKΔT^{CD4} mice with an anti-CD27L blocking antibody fails to restore T cell development

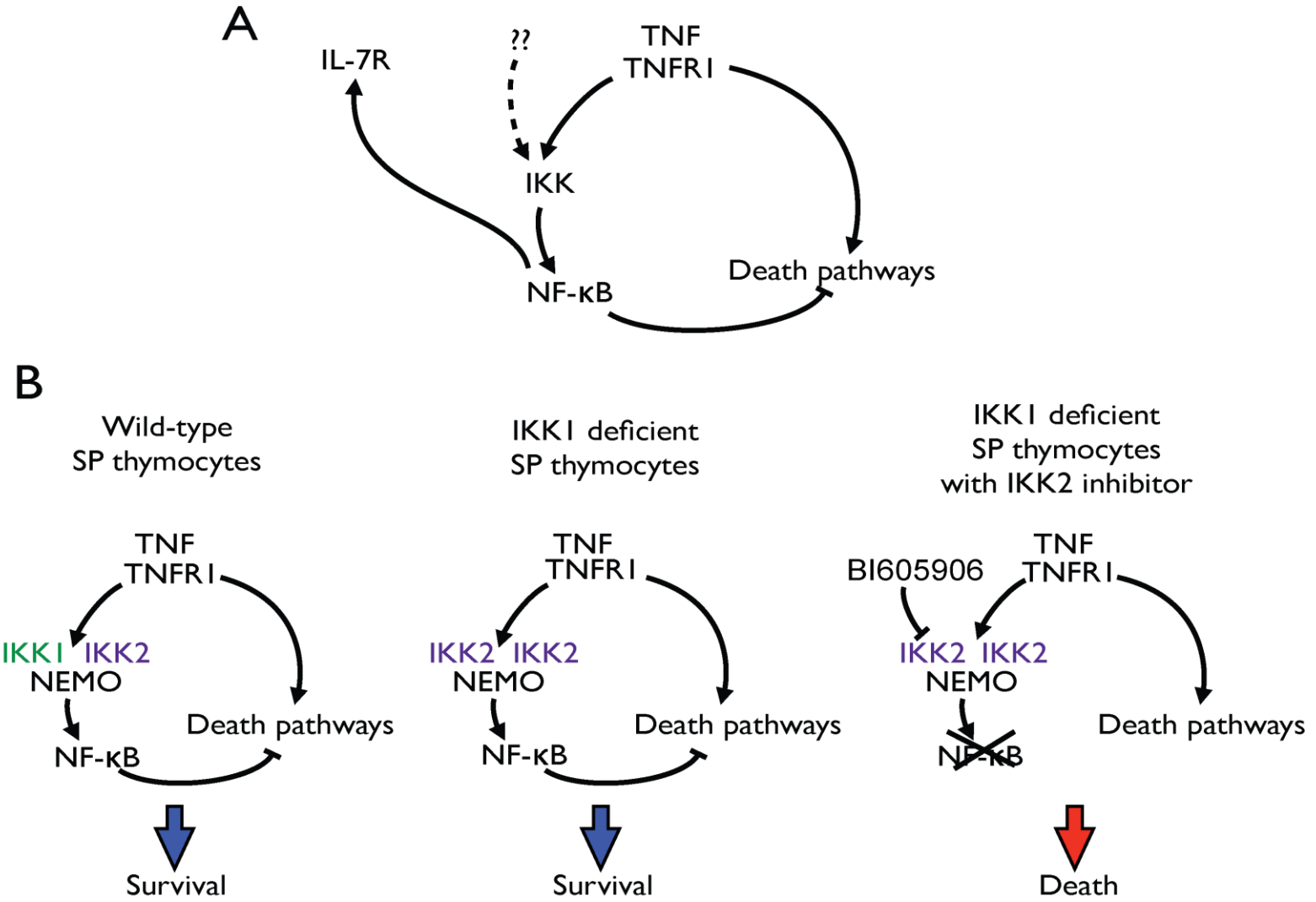
8-27 week old IKKΔT^{CD4} mice were treated over a 7 day period with an anti-CD27L blocking antibody or with an equivalent volume of PBS vehicle. Cre- (WT) littermates were left untreated. After the treatment period, mice were culled and cell numbers in the thymus (**A**) and periphery (LNs and spleen combined) (**B**) were calculated.

Cre- (WT), (n = 5); IKKΔT^{CD4} PBS treated, (n = 6); IKKΔT^{CD4} αCD27L treated, (n = 5). Data is shown from 2 independent experiments. Bar charts show mean cell number ± SEM. ns = not significant.


A
WT
IKKΔT^{CD4} PBS treated
IKKΔT^{CD4} αCD27L treated
DP
CD4 SP HSA^{hi}
CD4 SP HSA^{lo}
CD8 SP HSA^{hi}
CD8 SP HSA^{lo}
Cell no. (x 10^{-6})
ns
B
WT
IKKΔT^{CD4} PBS treated
IKKΔT^{CD4} αCD27L treated
Total T cells
CD4 naïve
CD8 naïve
Cell no. (x 10^{-6})
ns

Schematic 5.2 -Chapter 5 summary- The consequences of TNF signalling within SP thymocytes

(**A**) TNF works in an NF-κB dependent manner to upregulate IL-7R expression during the final stages of SP thymocyte maturation, just before the cells leave the thymus as recent thymic emigrants (Silva et al., 2014). However, it seems that TNF signalling is not essential for IL-7R upregulation, since naïve, peripheral T cells from *Tnfrsf1a*$^{-/-}$ mice or anti-TNF Ab treated WT mice have completely normal IL-7R expression. Our results indicate that other ligands, perhaps also belonging to the TNFSF, work in an IKK and NF-κB dependent manner to upregulate IL-7R expression on SP thymocytes.
(**B**) We used a combined genetic and pharmacological approach to acutely block NF-κB signalling in thymocytes. IKK1 deficient SP cells are no more sensitive to TNF induced death than their WT counterparts. This is most likely due to the formation of IKK2 homodimers that can compensate for the loss of the IKK1 subunit. However, addition of the IKK2 inhibitor, BI605906, to IKK1 deficient thymocytes *in vitro* caused much TNF induced cell death among all SP subsets. We speculate that this is due to a complete and acute block in NF-κB signalling. Hence, an acute block in NF-κB signalling is sufficient to render SP cells susceptible to TNF.


A
IL-7R
??
TNF
TNFR1
IKK
NF-κB
Death pathways
B
Wild-type
SP thymocytes
TNF
TNFR1
IKK1 IKK2
NEMO
Death pathways
NF-κB
Survival
IKK1 deficient
SP thymocytes
TNF
TNFR1
IKK2 IKK2
NEMO
Death pathways
NF-κB
Survival
IKK1 deficient
SP thymocytes
with IKK2 inhibitor
BI605906
TNF
TNFR1
IKK2 IKK2
NEMO
Death pathways
NF-κB
Death

Chapter 6 The mechanism of TNF induced death in developing T cells

6.1 Introduction

Numerous receptor families cause activation of the NF-κB pathway. Among them, the TNFR, TLR, IL-1R, and antigen receptor families have been extensively studied (Hayden and Ghosh, 2014). Of note, receptors that activate NF-κB signalling tend to lack any enzymatic activity of their own (Aggarwal, 2003; Hayden and Ghosh, 2014). For this reason, receptor signalling to NF-κB relies heavily on the recruitment of adaptor proteins. These adaptors are known for their protein:protein interaction domains, which facilitate the formation of huge multimeric protein complexes. Death domains (DD), receptor interacting protein (RIP) domains, and TNF receptor associated factor (TRAF) domains are all important mediators of ligand induced signalling to NF-κB (Hayden and Ghosh, 2014). The large, receptor associated complexes will eventually recruit proteins with enzymatic activity. Among them is the IKK complex, the activation of which leads to the nuclear translocation of NF-κB dimers.

The special relationship between TNF and NF-κB has long been recognised. TNF is a strong inducer of NF-κB activation and, in turn, NF-κB promotes TNF expression (Mercurio and Manning, 1999; Shakhov et al., 1990). TNFR1 is believed to be the main mediator of TNF's biological functions (Hayden and Ghosh, 2014; Vandenabeele et al., 1995). Binding of TNF to TNFR1 can result in activation of MAPK, apoptosis, and necroptosis pathways, in addition to the NF-κB pathway (Aggarwal, 2003; Vanden Berghe et al., 2014). TNF binding induces conformational changes on TNFR1. This allows recruitment of TRADD to the DD of TNFR1 (Hsu et al., 1995). TRADD is then able to recruit both RIPK1 and TRAF2 to the membrane (Hsu et al., 1996a; 1996b). It is believed that RIPK1 may aid TRADD in the recruitment of TRAF2 (Pobezinskaya et al., 2008). The resulting, multi-protein structure that forms around membrane-bound

TNFR1 is called complex I (Brenner et al., 2015; Hayden and Ghosh, 2014; **Schematic 6.1**). It is important for the downstream activation of NF-κB.

TRAF2 can recruit the IKK complex to complex I through a direct interaction with both IKK1 and IKK2 (Devin et al., 2001). However, TRAF2 knockout cells are not deficient in NF-κB activation (Yeh et al., 1997). This is believed to be due to compensation by TRAF5, since TRAF2/5 double knockout cells fail to activate NF-κB (Tada et al., 2001). RIPK1 also enables recruitment of the IKK complex to TNFR1. This occurs through the direct binding of RIPK1 to NEMO (Zhang et al., 2000). Interestingly, the kinase activity of RIPK1 seems to be dispensable for IKK recruitment (Lee et al., 2004). Hence, RIPK1 seems to adopt somewhat of a scaffold function when working towards the activation of NF-κB. A further function of RIPK1, in complex I, is thought to be the recruitment of TAK1 (Ea et al., 2006). TAK1 is an IKK kinase (IKK-K). Therefore, it is likely that RIPK1 facilitates the activation of the IKK complex by bringing it into close proximity to TAK1 (Ea et al., 2006). However, whilst some reports suggest that RIPK1 deficient cells are unable to activate NF-κB (Devin et al., 2001; Kelliher et al., 1998), others report normal IKK recruitment and NF-κB activation in the absence of RIPK1 (Wong et al., 2010). It has also been suggested that RIPK1 deficient cells are able to recruit the IKK complex (most likely through IKK1/2 binding to TRAF2), but fail in their ability to activate it (Devin et al., 2001).

Recently, there has been much evidence to suggest that ubiquitination of RIPK1 is required for activation of NF-κB signalling in response to TNF (Ea et al., 2006; Silke, 2011). It has been proposed that the lysine-63 (K63) linked ubiquitination of RIPK1, on lysine 377, is necessary for recruitment of both the IKK complex and TAK1 (Ea et al., 2006). Initially, it was believed that TRAF2 was directly responsible for attachment of the K63 ubiquitin chains to RIPK1 (Hayden and Ghosh, 2012). However, more recent work has indicated that the inhibitor of apoptosis proteins (IAPs), cIAP1 and cIAP2, are required for RIPK1 ubiquitination (Yin et al., 2009). The IAPs are defined by the presence of a baculovirus IAP repeat (BIR) domain. However, like TRAF2, the IAPs also possess really interesting new gene (RING) finger domains that are capable of

acting as E3 ligases and hence of inducing ubiquitination (Estornes and Bertrand, 2015). RIPK1 remains free of K63 ubiquitin chains in cIAP1/2 double knockout cells, or cells that have been treated with IAP antagonists (Mahoney et al., 2008; Varfolomeev et al., 2008). TRAF2/5 double knockout cells also show a similar absence of K63-linked ubiquitinated RIPK1 (Wong et al., 2010). Such evidence agrees with the hypothesis that TRAF2 may indeed be responsible for RIPK1 ubiquitination, but indirectly so, via the recruitment of cIAP1/2.

The cIAPs have also been shown to enable recruitment of the linear ubiquitin chain assembly complex (LUBAC) to complex I (Haas et al., 2009; Kirisako et al., 2006). The LUBAC is believed to consist of heme-oxidised iron regulatory protein 2 ubiquitin ligase 1 (HOIL-1), HOIL-1 interacting protein (HOIP), and SHANK-associated RH domain interactor (SHARPIN) (Gerlach et al., 2011; Kirisako et al., 2006). The LUBAC is thought to help stabilise TNFR1 complex I, resulting in sustained activation of NF-κB. It is able to add unusual linear chains of ubiquitin onto its substrates. One such substrate has been identified as NEMO. It seems that linear ubiquitination of NEMO, by the LUBAC, may result in direct activation of the IKK complex (Fujita et al., 2014). Ultimately, the cIAP proteins may facilitate NF-κB activation, not only via K63-linked ubiquitination of RIPK1, but also via LUBAC recruitment.

Eventually, and perhaps by several means, TNF induced signalling leads to activation of the IKK complex. IκBα is then phosphorylated and K48-linked ubiquitinated, marking it for degradation by the proteasome. Finally, NF-κB dimers can enter the nucleus, where they increase the transcription of both pro-inflammatory and anti-apoptotic genes. Cellular FLICE-like inhibitory protein (c-FLIP), TRAF1, cIAP1, and cIAP2 are among the anti-apoptotic proteins induced by NF-κB (Aggarwal, 2003). Importantly, the complex I mediated production of anti-apoptotic proteins is necessary to counteract against TNF induced cell death.

If complex I becomes destabilized then it may become endocytosed, resulting in formation of a new, cytosolic complex. Upon dissociation from the membrane,

TNFR1 remains connected to TRADD, TRAF2, and RIPK1. However, a variety of death signalling inducing signalling complex (DISC) proteins are additionally recruited. DISC proteins include TRADD, FADD, and TRAF1 (Hsu et al., 1996b; Pasparakis and Vandenabeele, 2015; Sedger and McDermott, 2014). The DISC proteins enable the formation of a scaffold, which recruits pro-caspase-8, an initiator caspase. This caspase-8 containing complex is termed complex IIa (Pasparakis and Vandenabeele, 2015; **Schematic 6.1**). In certain situations, RIPK3 may also be recruited, resulting in the formation of complex IIb (Pasparakis and Vandenabeele, 2015; **Schematic 6.1**). The circumstances leading to complex IIb formation are rare, but include TNF stimulation when IAPs are inhibited (for example with Smac mimetics), or when there is a deficiency of TAK1 or NEMO (Dondelinger et al., 2013; Legarda-Addison et al., 2009; Pasparakis and Vandenabeele, 2015; Wang et al., 2008). Formation of both complex IIa and complex IIb leads to apoptosis. Occasionally, complex IIb can undergo further changes to form complex IIc, also known as the necrosome. This occurs in the absence of caspase-8 activity and the presence of high levels of RIPK3 and its substrate, mixed lineage kinase like (MLKL) (Pasparakis and Vandenabeele, 2015; **Schematic 6.1**). Formation of the necrosome leads to a kinase-regulated form of necrotic cell death, known as necroptosis (Pasparakis and Vandenabeele, 2015).

The proteolytic cleavage of pro-caspase-8 into active caspase-8 is necessary for the induction of apoptosis. Pro-caspase-8 activation involves homodimerisation. This brings the catalytic cysteine regions of each subunit into close contact, thus enabling proteolytic cleavage to occur (Chang et al., 2003). Active caspase-8 is responsible for the activation of caspase-3, an executioner caspase. Caspase-3 mediates the release of caspase-activated DNAse (CAD), resulting in the degradation of genomic DNA and subsequent apoptotic cell death (Enari et al., 1998). Caspase-8 inhibits the formation of the necrosome. It reportedly does so via cleaving RIPK1, RIPK3, and cylindromatosis (CYLD, an enzyme that deubiquitinates RIPK1) (Feng et al., 2007; Lin et al., 1999; O'Donnell et al., 2011).

The protein c-FLIP is an important inhibitor of both apoptosis and necroptosis (Pasparakis and Vandenabeele, 2015). If expressed at high enough concentrations, c-FLIP can be recruited to the DISC via FADD (Safa, 2013). c-FLIP mimics the structure of pro-caspase-8 and thus acts as a competitive inhibitor (Pop et al., 2011). It can prevent the formation of the fully active caspase-8 enzyme by competing with pro-caspase-8 for recruitment to the DISC and also by forming a heterodimer with pro-caspase-8 (Pop et al., 2011). The method by which c-FLIP inhibits necroptosis is currently unclear, but has also been shown to involve its heterodimerisation with caspase-8 (Oberst et al., 2011).

Under normal conditions, cells are not susceptible to TNF induced death. Complex I mediated NF-κB activation and the production of anti-apoptotic/anti-necroptotic proteins counteracts against complex II mediated cell death. In **Chapters 4 and 5** we discovered that SP thymocytes, when highly deficient in NF-κB signalling, are very susceptible to TNF induced death. In addition, certain SP subsets were found to be more sensitive to TNF than others. The signalling events that occur downstream of TNFR1 are highly complex and remain incompletely understood. In **Chapter 6** we have attempted to more precisely determine the mechanism of TNF induced death in thymocytes in the absence of NF-κB signalling. Furthermore, we have investigated why certain SP subsets are intrinsically more sensitive to TNF than others.

6.2 Results

6.2.1 Ubiquitous expression of TNFR signalling components in thymocytes

We wished to determine why certain thymocyte populations are more susceptible to TNF induced death than others. As thymocytes mature through the DP and SP stages of development they undergo phenotype and functional changes and consequently, the expression patterns of a huge number of genes are altered. We hypothesised that, before thymocytes reach a certain stage of maturity, they may lack expression of key components required for signalling downstream of the TNFR. These differences may explain why, in the absence of NF-κB signalling, some subsets are more susceptible to TNF induced death than others.

Previously in the laboratory, RNA sequencing analysis was performed on DP1, DP2, DP3, total CD8SP, CD4SP HSA^{hi}, and CD4SP HSA^{lo} thymocyte populations (Sinclair et al., 2013). We therefore took advantage of this resource to investigate expression of key adaptors and kinases downstream of TNFR signalling. As predicted, many genes were differentially expressed between the different thymocyte subsets. We concentrated on genes related to signalling downstream of TNFR1. These included genes connected with NF-κB pathways, genes for receptors and adaptors, and genes involved in the inhibition or induction of cell death (**Figure 6.1**). Surprisingly, the expression of many TNF signalling related genes did not change dramatically as the thymocytes matured (**Figure 6.1**). This suggests that even the DP1 thymocytes, the least mature population examined, are capable of normal TNF mediated signal transduction. Interestingly, however, the genes for TRAF1, RIPK1, caspase-8, and cIAP2 were all upregulated between the DP and SP stages (**Figure 6.1**).

As a control, we looked at expression of *Il7r* (the gene encoding IL-7Rα). IL-7R expression is highly regulated during thymocyte development. Although present during the DN stage of development, the IL-7R is absent on DP thymocytes

(Sudo et al., 1993). Cells re-express the IL-7R during the SP stages of development, following successful positive selection (Sinclair et al., 2011). As expected, *Il7r* was undetectable within DP1 thymocytes, but upregulated in SP thymocytes.

6.2.2 TRAF1, TRAF2, cIAP1, and cIAP2 mRNA is increased in response to TNF stimulation

When TNF binds to TNFR1, downstream signalling cascades are initiated. This is likely to involve increased transcription of genes and increased protein turnover. By determining which genes are upregulated by TNF, we hoped to better understand the TNF signalling pathway. TCR^{hi}CD8SP thymocytes were sorted from the thymii of F5 *Rag1*$^{-/-}$ mice. The sorted cells were then cultured for 4hr in medium supplemented with either TNF cytokine or PBS vehicle. RNA sequencing was performed on the cultured cells and also on uncultured cells, taken directly after sorting. TNF was found to upregulate the expression of a small number of genes encoding for components downstream of TNFR1 (**Figure 6.2A**). Notably, the genes for TRAF1, TRAF2, cIAP1, and cIAP2 were all highly upregulated in the CD8SP thymocytes by 4hr of culture with TNF (**Figure 6.2A**).

As a control, we looked at expression of *Il7r*. IL-7 induced signalling suppresses the transcription of its receptor (Park et al., 2004). Conversely, when T cells are cultured in the absence of IL-7, expression of the IL-7R is increased. Furthermore, culture of SP thymocytes with TNF is known to induce expression of the IL-7R (Silva et al., 2014; **Chapter 5**). As expected, culture of the TCR^{hi}CD8SP F5 thymocytes for 4hr in the absence of IL-7 did increase transcription of *Il7r* (**Figure 6.2A**). However, mRNA levels were increased even further when cells were cultured with TNF (**Figure 6.2A**). These data identify TRAF1, TRAF2, cIAP1, cIAP2, IL-7R, and members of the Rel family as key NF-κB targets downstream of TNFR.

6.2.3 Gene expression changes in the absence of IKK signalling *in vivo*

We wished to determine why IKK deficient, SP thymocytes are far more susceptible to TNF induced death than WT, SP thymocytes. We hypothesised that it may be due to reduced expression of certain NF-κB dependent inhibitors of apoptosis by the IKK deficient cells. To test this hypothesis, we sorted $TCR^{hi}CD8SP$ thymocytes from *Tnfrsf1a*$^{-/-}$ $IKK\Delta T^{CD4}$ mice and *Tnfrsf1a*$^{-/-}$ littermates. RNA sequencing was then performed on the sorted cells.

As expected, the *Tnfrsf1a*$^{-/-}$ $IKK\Delta T^{CD4}$ thymocytes had significantly reduced transcription of the genes for many Rel family members, (**Figure 6.2B**), consistent with our observations that RelB, p105, c-Rel, and p100 are themselves NF-κB targets in thymocytes (**Figure 6.2A**). Furthermore, *Il7r* mRNA was much lower in the IKK deficient cells (**Figure 6.2B**). The expression of the gene for CYLD, a protein that negatively regulates NF-κB signalling (Sun, 2010), was also reduced in the IKK deficient cells (**Figure 6.2B**). NF-κB signalling is known to increase the production of TRAF1, TRAF2, cIAP1, and cIAP2 (Wang et al., 1998). Consistent with this view, we observed that the genes encoding these proteins were significantly downregulated in the IKK deficient thymocytes (**Figure 6.2B**). The reduced expression of the cIAP and TRAF genes is of particular interest, since the protein products of these are inhibitors of apoptosis (Wang et al., 1998). These data suggest that reduced production of cIAPs and TRAFs may contribute to the increased sensitivity of IKK deficient thymocytes to TNF induced death.

6.2.4 The *in vitro* inhibition of cIAP1 and cIAP2 increases death among SP thymocytes

We hypothesised that the inhibition of cIAP1 and cIAP2 would increase death among SP thymocytes. Smac mimetics are compounds designed to mimic the N-terminal tetrapeptide of Smac, an endogenous IAP antagonist (Wu et al., 2007). They have been developed for use in the treatment of cancer, to

counteract the apoptotic resistance of cancer cells that is often caused by overproduction of IAPs (Wu et al., 2007). Birinapant is a bivalent Smac mimetic that has been shown to cause degradation of cIAP1 and cIAP2 in intact cells and to cause formation of a RIPK1:caspase-8 complex (Benetatos et al., 2014).

Suspensions of whole, mashed thymii from WT mice were pre-cultured overnight (16hr) with either TNF cytokine or PBS vehicle. The thymocytes were then cultured for a further 5hr with varying concentrations of either birinapant or DMSO vehicle. Birinapant induced death in each SP population by 5hr in a dose dependent manner (**Figure 6.3**). Very similar results were obtained when cell culture was performed using an alternative Smac mimetic, GDC-0152 (data not shown). Expression of *Birc3*, the gene encoding cIAP2, was found to be developmentally regulated among thymocytes (**Figure 6.1**). For this reason, we considered that different SP subsets may have a different abundance of IAPs and therefore, may be more or less susceptible to Smac mimetic induced death. *Birc3* expression was highest among CD4SP HSA^{lo} cells (**Figure 6.1A**). However, the susceptiblility of this subset towards birinapant was not significantly different from that of other subsets (**Figure 6.3**). TNF signalling leads to increased production of cIAP1/2 (Wang et al., 1998; **Figure 6.2A**). It was therefore possible that the TNF stimulation of thymocytes would induce higher levels of cIAP1/2, rendering the thymocytes less susceptible to Smac mimetic induced cell death. We therefore pre-stimulated thymocytes with TNF prior to culture with birinipant. Comparing susceptibility of different subsets to birinapant induced cell death did not reveal any significant differences between TNF pre-treated and control cultures (**Figure 6.3**).

These data do indicate that the IAP antagonist birinapant induces cell death among SP thymocytes (**Figure 6.3**). As to whether certain SP subsets or TNF, as opposed to PBS, treated thymocytes have different responses towards IAP inhibition, remains to be fully explored.

6.2.5 TNFR1 is expressed at similar levels among different SP thymocyte populations

We hypothesised that the level of TNFR1 expression may vary between different SP thymocyte populations and that this may explain the increased susceptibility of certain SP subsets towards TNF induced death in the absence of IKK expression. We were unable to detect expression of TNFR1 on thymocytes by FACS. Therefore, we measured TNFR1 indirectly by biological inhibition of TNF induced death with a blocking mAb.

Thymocytes from huCD2^{iCre} *Ikk1*$^{fx/fx}$ R26R^{EYFP} mice were pre-incubated with a titration of blocking antibody towards TNFR1 for 2hr. The IKK2 inhibitor, BI605906 (10μM) was then added to the cells, along with TNF cytokine (30ng/mL), and TNF induced cell death assessed at 24hr. In the absence of any TNFR1 blocking Ab, the thymocytes showed poor viability, while high concentrations successfully blocked TNF activity (**Figure 6.4**). We therefore calculated the IC_{50} of TNFR1 blocking Ab for the different SP subsets. These values represented the concentration of TNFR1 blocking Ab required to reduce maximum TNF induced cell death in the thymocyte population by 50%. The IC_{50} values were very similar between all SP subsets. They were as follows: CD8SP HSA^{lo}, 9.7μg/mL; CD8SP HSA^{hi}, 8.9 μg/mL; CD4SP HSA^{hi}, 6.9μg/mL; CD4SP HSA^{lo}, 1.5μg/mL (**Figure 6.4**). The CD4SP HSA^{lo} population did have a slightly lower IC_{50} value suggesting that this subset, if anything, has a lower level of TNFR1. However, it appears that expression of TNFR1 does not differ substantially between SP subsets and is therefore unlikely to account for the differences in TNF susceptibility.

6.2.6 Expression of RIPK1 is developmentally regulated among thymocytes

The results of RNA sequencing had revealed that the gene *Ripk1* is developmentally regulated among thymocytes (**Figure 6.1**). RIPK1 is of particular importance in the TNF signalling pathway, as it is required for the

formation of both complex I and complex II (Christofferson et al., 2014; Festjens et al., 2007). In wild-type cells, RIPK1 is required to activate NF-κB signalling and promote survival. However, it is also an obligate component of complex II, in which it initiates apoptosis or necroptosis. Changes in the abundance of RIPK1 could therefore explain the differing sensitivities of NF-κB deficient thymocyte populations towards TNF induced cell death.

We wished to determine whether expression levels of the gene for RIPK1 correlated with those for the protein product. Intracellular staining for RIPK1 was performed on thymocyte suspensions obtained from WT and *Tnfrsf1a*$^{-/-}$ IKKΔT^{CD4} mice. Flow cytometry was then used to compare the levels of RIPK1 between the different thymocyte populations. Importantly, within the different DP and SP subsets examined, a correlation was found between the abundance of RIPK1 protein and mRNA (**Figure 6.5B**). Furthermore, the SP thymocyte populations with the highest expression levels of RIPK1 protein/mRNA were found to be those that, in the absence of NF-κB activation, exhibited the highest sensitivities towards TNF induced cell death (**Figure 6.5**; **Chapter 5**). Since RIPK1 expression changes substantially during the DP and SP stages of thymocyte development, we also examined its abundance in DN thymocytes. Interestingly, RIPK1 expression is high during DN1, increasing further as cells progress into DN2 (**Figure 6.5**). Between DN2 and DN3, expression falls (**Figure 6.5**). At DN4 cells appear heterogeneous in their RIPK1 expression, with a small population expressing very low/undetectable levels (**Figure 6.5A**). The fact that levels of RIPK1 change between different thymocyte populations implies that this protein may play a role in T cell development.

Of note, no differences in RIPK1 expression were apparent between the WT and *Tnfrsf1a*$^{-/-}$ IKKΔT^{CD4} mice (**Figure 6.5B**). *Ripk1* expression changed neither when thymocytes were TNF stimulated (**Figure 6.2A**) nor in the absence of IKK1 and IKK2 expression (**Figure 6.2B**). However, whether an NF-κB deficient SP thymocyte dies or survives in response to TNF, could be highly dependent on the abundance of cytoplasmic RIPK1.

6.2.7 The RIPK1 inhibitor, necrostatin-1 protects NF-κB deficient SP thymocytes from TNF induced death

We hypothesised that the inhibition of RIPK1 would rescue NF-κB deficient SP thymocytes from TNF induced death**.** We tested this using necrostatin-1, an allosteric inhibitor of RIPK1 kinase activity (Degterev et al., 2008). Thymocytes from huCD2^{iCre} *Ikk1*$^{fx/fx}$ R26R^{EYFP} mice were pre-incubated for 2hr with varying concentrations of necrostatin-1. Following this, the thymocytes were cultured for a further 21hr in the presence of the IKK2 inhibitor, BI605906 (10μM), plus either TNF cytokine (30ng/mL) or H_2O vehicle (**Figure 6.6A**). As expected, in the absence of any necrostatin-1, the IKK deficient thymocytes underwent substantial TNF induced death (**Figure 6.6A**). However, as the concentration of necrostatin-1 increased, the percentage of dead cells within each SP thymocyte population decreased (**Figure 6.6A**). Calculation of the IC_{50} values for the different SP subsets revealed the concentration of necrostatin-1 required to decrease TNF induced death by 50% (**Figure 6.6A**). The CD8SP HSA^{lo} population had the highest IC_{50} value, consistent with FACS data suggesting that this subset contains a greater abundance of RIPK1 protein (**Figure 6.6A**; **Figure 6.5**).

RIPK1 is required for activation of caspase-8 (Wang et al., 2008). We therefore asked whether necrostatin-1 decreased active caspase-8. 4hr after the addition of IKK2 inhibitor ±TNF, the IKK deficient CD8SP HSA^{lo} cells were assessed for viability and also for active caspase-8 (**Figure 6.6B**). As the concentration of necrostatin-1 increased, the percentage of both dead cells and also active caspase-8^{+} live cells decreased (**Figure 6.6B**). This suggests that necrostatin-1 rescued the cells from caspase-8 dependent apoptosis.

To test the requirement for RIPK1 kinase activity in NF-κB activation, we investigated IL-7R expression on necrostatin-1 treated thymocytes. Following a 5hr pre-incubation with varying concentrations of necrostatin-1, huCD2^{iCre} *Ikk1*$^{fx/fx}$ R26R^{EYFP} thymocytes were cultured for a further 16hr with or without TNF (**Figure 6.6C**). After the culture period, cells were assessed for their

expression of the IL-7R. As expected, cells upregulated their IL-7R expression when in the presence of TNF (**Figure 6.6C**). However, necrostatin-1 was found to have no effect on IL-7R expression (**Figure 6.6C**). These results further support the view that RIPK1 kinase activity is not necessary for NF-κB activation (Lee et al., 2004).

6.2.8 Evidence that TNF induced death is caspase-8 dependent

In the absence of IKK signalling, thymocytes are susceptible to TNF induced cell death. However, whether this cell death is mediated by caspase-8 dependent apoptosis or caspase-independent necroptosis remains to be determined. To investigate this we made use of the caspase-8 specific inhibitor, Z-IETD-FMK.

Thymocytes from huCD2^{iCre} *Ikk1*$^{fx/fx}$ R26R^{EYFP} mice were pre-incubated for 1hr with either medium alone or medium containing Z-IETD-FMK (40μM). The cells that had been pre-incubated in medium were then cultured for 4hr with TNF cytokine (30ng/mL) plus either DMSO vehicle or the IKK2 inhibitor, BI605906 (10μM). Alternatively, cells that had been pre-incubated with Z-IETD-FMK were cultured for 4hr with TNF cytokine (30ng/mL) plus both BI605906 (10μM) and Z-IETD-FMK (20μM). Finally, the cells were assessed for the presence of active caspase-8 and for viability.

When the IKK1 deficient thymocytes were cultured with IKK2 inhibitor and TNF they stained more highly for active caspase-8 and for cell death than when they were cultured with DMSO and TNF (**Figure 6.7**). The increased death in the presence of the IKK2 inhibitor was expected, given our previous results (**Chapter 5**). The presence of active caspase-8 correlated well with staining for cell death, thus indicating that the TNF induced death is likely to be caspase-8 dependent (**Figure 6.7**). When present in the culture, the caspase-8 inhibitor, Z-IETD-FMK was able to reduce both active caspase-8 and cell death among many of the thymocyte populations (**Figure 6.7**). Again this indicates that the induced death is likely caspase-8 dependent. Surprisingly, Z-IETD-FMK failed

to decrease active caspase-8 and cell death among the CD8SP HSA^{lo} and CD4SP HSA^{lo} thymocytes (**Figure 6.7**). As a control for Z-IETD-FMK, the negative control for caspase inhibitors, Z-FA-FMK was added to some of the cells. This was found to have no inhibitory effect on either the percentage of active caspase-8 or on cell death (data not shown). In conclusion, it seems that the TNF induced death of NF-κB deficient cells is caspase-8 dependent and therefore due to apoptosis rather than necroptosis (Pasparakis and Vandenabeele, 2015).

6.3 Discussion

In **Chapter 6** we aimed to understand the mechanism of TNF induced death that occurs in SP thymocytes in the absence of NF-κB signalling. We began by investigating expression levels of genes that are known to encode for signalling components downstream of TNFR1. We observed the changes in gene expression that occur in thymocytes: during development, upon stimulation with TNF, and upon loss of IKK. We compared the genes that were up/downregulated in each scenario. In this way, we were able to better understand which components of the TNF signalling pathway were of particular relevance in IKK deficient thymocytes.

In **Chapter 5** we confirmed that different SP subsets are intrinsically more or less susceptible to TNF induced death. We found that, when thymocytes were deficient in IKK or expressed a dominant negative IκBα, the CD8 lineage showed greater susceptibility to TNF than the CD4 lineage. Within each lineage, the HSA^{lo} cells were more susceptible than were the HSA^{hi} cells. The production of CD8SP thymocytes is a slower process than that of CD4SP thymocytes (Saini et al., 2010), and HSA^{lo} cells are developmentally more mature than HSA^{hi} cells (Tian et al., 2001). Hence, it seems that NF-κB deficient thymocytes become more susceptible to TNF induced death as they mature. We hypothesised that this may be due to a lack of expression of TNF signalling components during the earlier stages of SP thymocyte development. However, the expression of many TNF signalling related genes did not change during the DP and SP stages of thymocyte maturation (**Figure 6.1**). These included the genes encoding for the important adaptors TRADD, FADD and TRAF2 (**Figure 6.1**). It appeared that even the less mature thymocytes possessed the cellular machinery required for the transduction of TNF signalling.

Interestingly, expression of the genes encoding RIPK1, TRAF1, cIAP2, caspase-8, and certain NF-κB family members did change during thymocyte development (**Figure 6.1**). The IAPs and TRAFs have been implicated in the suppression of TNF induced apoptosis (Wang et al., 1998). Their production is

known to be induced by NF-κB (Wang et al., 1998). Indeed, in the absence of both IKK1 and IKK2, expression of the genes encoding TRAF1, TRAF2, cIAP1, and cIAP2 was significantly downregulated (**Figure 6.2B**). Moreover, in WT cells, TRAF1 and IAP expression was hugely increased upon stimulation with TNF, most probably due to increased NF-κB transcriptional activity (**Figure 6.2A**). As WT thymocytes mature, they increase their expression of the genes for TRAF1 and cIAP2 (**Figure 6.1**). Under normal physiological conditions, the more mature SP thymocytes may therefore rely more heavily on TRAF1 and cIAP2 expression for survival. This may explain why, in the absence of IKK, it is these mature cells that become the most TNF susceptible. Of note, among WT thymocytes it is only DP and CD4SP HSA^{hi} populations that show any susceptibility to TNF induced death, and this is only at a low level. We speculate that this could be due to the lower expression levels of TRAF1 and/or cIAP2 and/or NF-κB among these populations (**Figure 6.1**).

Expression of *Tnfrsf1a*, the gene for TNFR1, was found to change slightly between the DP and SP stages of thymocyte development (**Figure 6.1**). Interestingly, expression was highest within the DP and CD4SP HSA^{hi} populations (**Figure 6.1**). Among wild-type thymocytes, it is these populations that show the greatest sensitivity towards TNF induced death. We hypothesised that this could perhaps be due to their slightly higher expression of TNFR1. We therefore wished to compare expression of the TNFR1 protein between different SP thymocyte subsets. We did this by use of an anti-TNFR1 blocking Ab. The concentration of blocking Ab required for a 50% rescue of IKK deficient thymocytes from maximum TNF induced death was calculated. It was found to be similar for all SP subsets, suggesting that TNFR1 expression varied very little (**Figure 6.4**). Ultimately, we concluded that the small changes in TNFR1 expression between SP cells were unlikely to explain their differing susceptibilities towards TNF induced death. Hence, we looked elsewhere for an explanation.

The developmental changes in the expression of *Ripk1* were of particular interest. RIPK1 is a crucial mediator of the life versus death decisions a cell makes in response to TNF (Christofferson et al., 2014; Festjens et al., 2007). In

the absence of NF-κB signalling, RIPK1 induces complex II mediated cell death. Expression levels of *Ripk1* were lower among DP cells and CD4SP HSA^{hi} cells, but higher among total CD8SP cells and CD4SP HSA^{lo} cells (**Figure 6.1**). Using flow cytometry, we found that levels of RIPK1 mRNA correlated well with abundance of the protein (**Figure 6.5B**). *Tnfrsf1a*$^{-/-}$ IKKΔT^{CD4} thymocytes were found to contain the same amount of RIPK1 protein as WT thymocytes (**Figure 6.5B**), suggesting that it is neither a TNF nor NF-κB regulated gene, and this was confirmed in our analysis of TNF induced gene expression changes (**Figure 6.2**). Hence, evidence suggests that increased expression of RIPK1 during development may account for increased sensitivity of IKK deficient cells towards TNF.

It is interesting to note that, throughout the DN stage of thymocyte development, the expression of RIPK1 protein changed dramatically (**Figure 6.5**). The levels of RIPK1 present at DN1 and DN2 were particularly high (**Figure 6.5**). By DN3 the abundance of RIPK1 was decreased slightly, and by DN4 expression was heterogeneous, with some cells expressing very low levels comparable to those of DP thymocytes (**Figure 6.5**). Throughout the DP1-3 stages, RIPK1 expression remained low (**Figure 6.5**). In IKKΔT^{CD2} mice, deletion of the IKK1 and IKK2 genes begins at the DN2 stage of thymocyte development. Upon investigation, we found that IKKΔT^{CD2} mice contained normal numbers of DN3 and DN4 thymocytes (**Chapter 3**). It is possible that, in IKK deficient mice, the low level of RIPK1 expression in DN4, DP, and CD4SP HSA^{hi} thymocytes is protecting these populations from TNF induced death. We speculate that, if DN1 or DN2 populations were made deficient in IKK1 and IKK2, they may be susceptible to TNF induced death mediated by their high expression levels of RIPK1.

We investigated the importance of RIPK1 kinase activity in TNF induced death using necrostatin-1, an allosteric inhibitor of RIPK1 kinase activity (Degterev et al., 2008). Necrostatin-1 was able to completely rescue IKK deficient thymocytes from TNF induced death (**Figure 6.6A**). Moreover, a higher concentration of necrostatin-1 was required to rescue CD8SP HSA^{lo} cells (which contained the greatest abundance of RIPK1 protein) and a lower

concentration was required to rescue CD4SP HSA^{hi} cells (which contained the lowest abundance of RIPK1 protein) (**Figure 6.6A**; **Figure 6.5**). This further confirmed that the TNF induced death of IKK deficient thymocytes was indeed RIPK1 mediated. Interestingly, necrostatin-1, in addition to reducing cell death, was also able to reduce the presence of active caspase-8 (**Figure 6.6B**). Furthermore, the caspase-8 inhibitor, Z-IETD-FMK was able to reduce the amount of cell death and the amount of active caspase-8 among TNF treated, IKK deficient thymocytes (**Figure 6.7**). Ultimately, when NF-κB signalling is inhibited, TNF induced death seems to be highly caspase-8 dependent (**Figure 6.6B; Figure 6.7**). This suggests that death is occurring via apoptosis, as opposed to necroptosis (Pasparakis and Vandenabeele, 2015).

Previous studies have shown the importance of RIPK1 in the TNFR1 complex I induced activation of IKK. However, the kinase activity of RIPK1 is thought to be dispensable for IKK recruitment (Lee et al., 2004). From previous studies and our earlier results, we know that expression of the IL-7R is a very good indicator of the strength of NF-κB signalling (Miller et al., 2014; Silva et al., 2014; **Chapter 3**). Necrostatin-1, even at high concentrations, failed to decrease IL-7R expression on SP thymocytes (**Figure 6.6C**). This suggests that NF-κB signalling is unaffected by the presence of necrostatin-1. This result was to be expected, given that necrostatin-1 inhibits only the kinase activity of RIPK1 and should not, therefore, interfere with NF-κB signalling (Degterev et al., 2008). Evidence suggests that TNF induced apoptosis mediated by complex IIa requires neither RIPK1 nor RIPK1 kinase activity (Ofengeim and Yuan, 2013). However, apoptosis, mediated by complex IIb, and necroptosis, mediated by the necrosome, are both reported to rely on RIPK1 kinase activity (Berger et al., 2014; Ofengeim and Yuan, 2013; **Schematic 6.1**). Necrostatin-1 was able to decrease caspase-8 dependent cell death among TNF treated, IKK deficient thymocytes (**Figure 6.6B**). Therefore, such cell death is likely apoptotic and mediated by complex IIb.

RNA sequencing had revealed that transcription of the genes for cIAP1 and cIAP2 could be TNF induced and was NF-κB dependent (**Figure 6.2**). cIAP proteins are important inhibitors of TNF induced death. Hence, we believed that

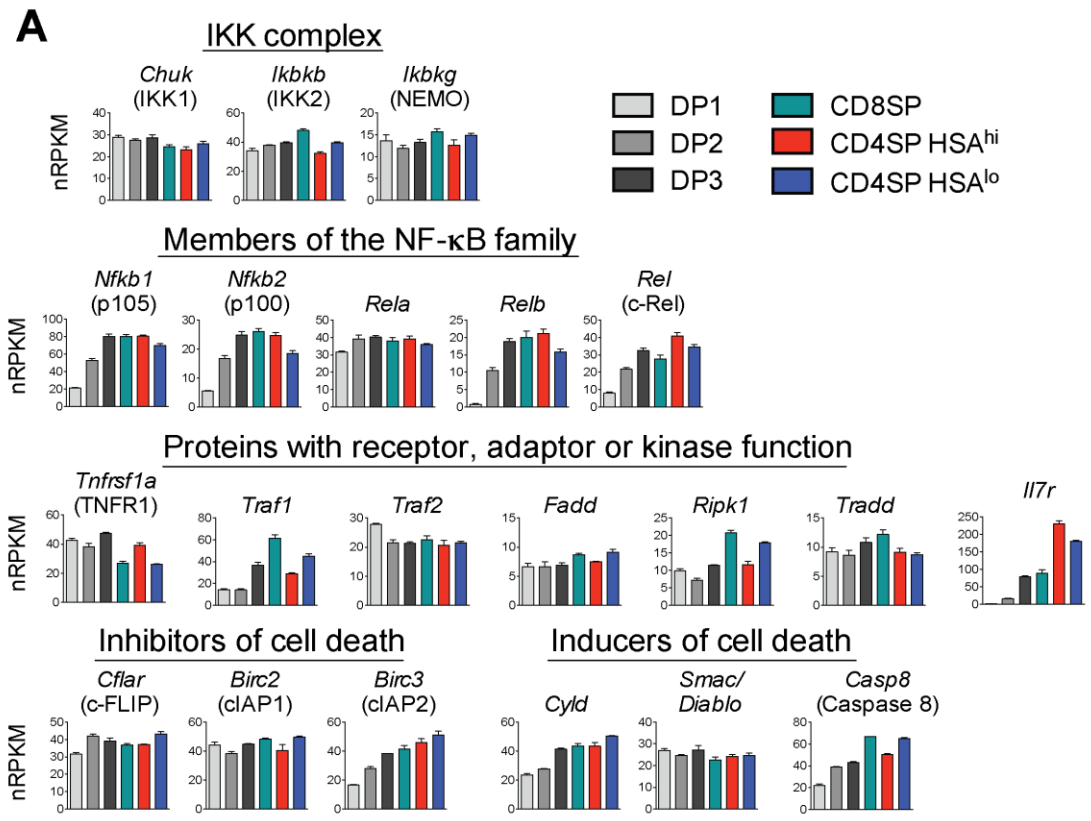
IKK deficient thymocytes may be susceptible to TNF induced death due to their lack of cIAPs. We cultured the Smac mimetic (IAP antagonist), birinapant with WT thymocytes. Birinapant has been shown to inhibit both cIAP1 and cIAP2 (Benetatos et al., 2014). Birinapant did cause dramatic death among the WT thymocytes, but only when used at very high concentrations (**Figure 6.3**). Previous studies have shown that, when used in conjunction with TNF, birinapant induces a greater amount of death (Benetatos et al., 2014; Wu et al., 2007). However, in our cultures, birinapant induced death was not increased by the presence of TNF (**Figure 6.3**). For this reason, we cannot definitively state that lack of cIAPs results in the TNF induced death of thymocytes.

In addition to the cIAPs, many other NF-κB targets have been identified as being important for protecting cells from TNF induced death. These include A1 (Bfl1), TRAF1, TRAF2, c-FLIP, and the X-chromosome linked IAP (XIAP) (Conte et al., 2001; Karin and Lin, 2002; Stehlik et al., 1998; Wang et al., 1998). However, in thymocytes we found that TNF caused strong induction of only a small number of anti-apoptotic genes, notably those encoding for TRAF1 and the cIAPs (**Figure 6.2A**).

In **Chapter 6** we used a variety of techniques to investigate the mechanisms behind TNF induced death. Transcriptomic analysis revealed the cIAPs and TRAFs to be key targets of NF-κB dependent TNF signalling (**Figure 6.2**). Although further evidence is required, we suspect that reduced production of these anti-apoptotic proteins may account for the increased susceptibility of IKK deficient thymocytes towards TNF. In previous chapters, we have shown that different thymocyte populations have different sensitivities to TNF. In IKK deficient cells, this is likely due to the developmental regulation of RIPK1 (**Figure 6.5**). Furthermore, it seems that the TNF induced death observed among IKK deficient thymocytes is caspase-8 dependent (**Figure 6.6B**; **Figure 6.7**). Hence, cell death is likely apoptotic, as opposed to necroptotic.

Figure 6.1 During the DP and SP stages of thymocyte development, the expression of many TNF signalling related genes remains fairly constant

(**A**) Thymii were taken from C57BL/6 wild-type mice. Single thymocyte suspensions were passed through the FACS machine and DP1, DP2, DP3, CD8SP, CD4SP HSA^{hi}, and CD4SP HSA^{lo} populations were sorted. Following correct sample processing, RNA sequencing was then performed. Cell sorting, RNA extraction, and preparation of samples for RNA sequencing were all performed by Charles Sinclair. Cluster generation on the Illumina high throughput sequencer was performed by members of the NIMR's RNA sequencing facility. nRPKM = number of reads per kilobase of exon per million sequenced reads. Bar charts show data from 3 independent cell sorts. The mean nRPKM ± SEM is indicated. (**B**) A diagram illustrating key components downstream of TNFR1. For many of these components, gene expression changes between the DP and SP stages of thymocyte development. This is indicated by the colour the component appears on the diagram.


A
IKK complex
Chuk (IKK1)
Ikbkb (IKK2)
Ikbkg (NEMO)
nRPKM
DP1
DP2
DP3
CD8SP
CD4SP HSAhi
CD4SP HSAlo
Members of the NF-κB family
Nfkb1 (p105)
Nfkb2 (p100)
Rela
Relb
Rel (c-Rel)
Proteins with receptor, adaptor or kinase function
Tnfrsf1a (TNFR1)
Traf1
Traf2
Fadd
Ripk1
Tradd
Il7r
Inhibitors of cell death
Cflar (c-FLIP)
Birc2 (cIAP1)
Birc3 (cIAP2)
Inducers of cell death
Cyld
Smac/ Diablo
Casp8 (Caspase 8)

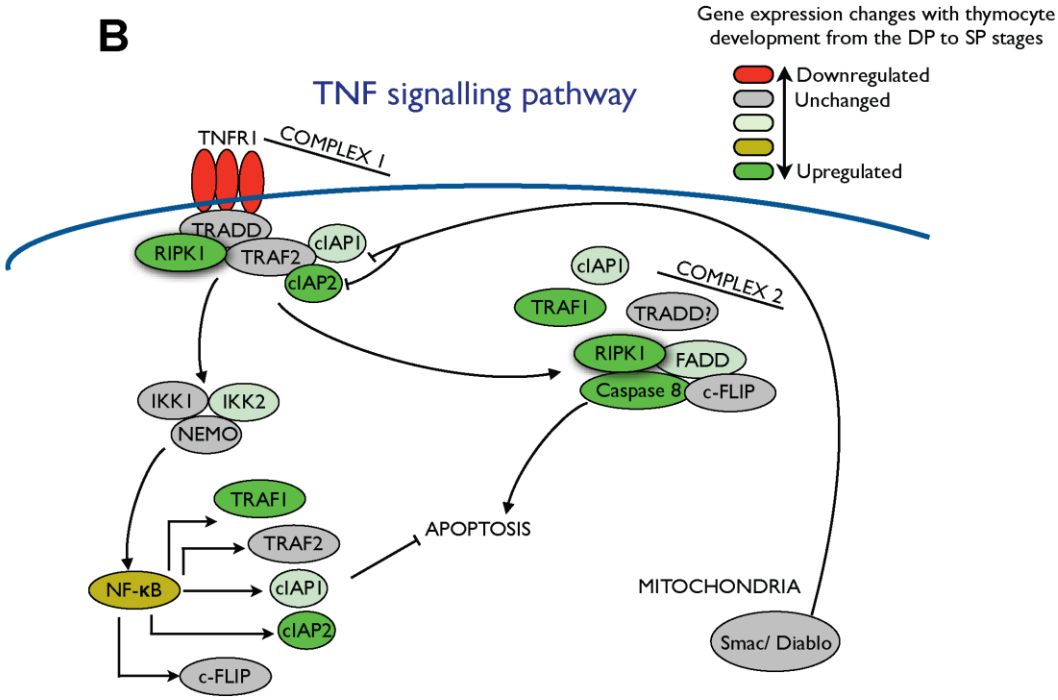

B
Gene expression changes with thymocyte development from the DP to SP stages
Downregulated
Unchanged
Upregulated
TNF signalling pathway
TNFR1
COMPLEX I
TRADD
RIPK1
TRAF2
cIAP1
cIAP2
IKK1
IKK2
NEMO
NF-κB
TRAF1
TRAF2
cIAP1
cIAP2
c-FLIP
APOPTOSIS
COMPLEX 2
TRADD?
FADD
Caspase 8
c-FLIP
MITOCHONDRIA
Smac/ Diablo

Figure 6.2 Expression of the genes encoding for the cIAPs and TRAFs is TNF inducible and NF-κB dependent

RNA sequencing was performed on $TCR^{hi}CD8SP$ thymocytes. (**A**) Thymii were taken from F5 *Rag1*$^{-/-}$ mice. These were then passed as a single cell suspension through a FACS machine, and a pure population of $TCR^{hi}CD8SP$ thymocytes was collected. The sorted cells were then cultured for 4hr in medium containing either PBS vehicle (PBS 4hr) or 30ng/mL TNF cytokine (TNF 4hr). Following culture, RNA was extracted from the cells. RNA was also extracted from some of the cells immediately following the sort (0hr). 3 independent cell sort and culture experiments were performed. Following correct preparation of the RNA samples, sequencing was performed using the Illumina high throughput sequencer. Cluster generation on the Illumina high throughput sequencer was performed by members of the NIMR's RNA sequencing facility. (**B**) Thymii were taken from *Tnfrsf1a*$^{-/-}$ mice and *Tnfrsf1a*$^{-/-}$ IKKΔT^{CD4} littermates. A pure population of $TCR^{hi}CD8SP$ cells was obtained via cell sorting. RNA was then extracted from the sorted cells. Four independent cell sorts were performed. Samples were then processed and their RNA sequenced. Preparation of RNA samples for sequencing and cluster generation on the Illumina high throughput sequencer were performed by members of the NIMR's RNA sequencing facility.

nRPKM = number of reads per kilobase of exon per million sequenced reads. The mean nRPKM ± SEM is indicated. ns = not significant; * = significant at $P<0.05$; ** = significant at $P<0.01$; *** = significant at $P<0.001$.

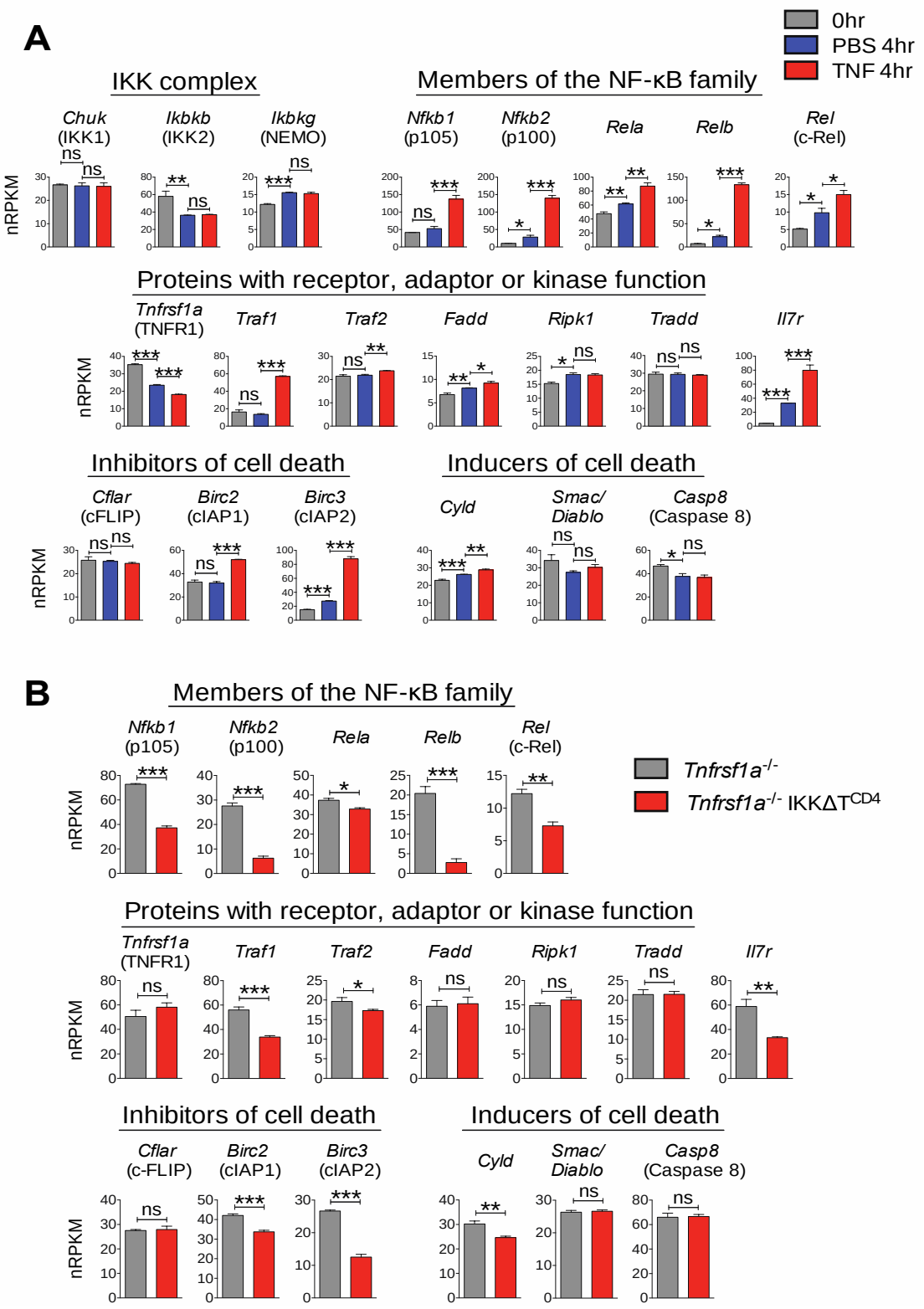

A
0hr
PBS 4hr
TNF 4hr
IKK complex
Members of the NF-κB family
Chuk (IKK1)
Ikbkb (IKK2)
Ikbkg (NEMO)
Nfkb1 (p105)
Nfkb2 (p100)
Rela
Relb
Rel (c-Rel)
nRPKM
Proteins with receptor, adaptor or kinase function
Tnfrsf1a (TNFR1)
Traf1
Traf2
Fadd
Ripk1
Tradd
Il7r
Inhibitors of cell death
Inducers of cell death
Cflar (cFLIP)
Birc2 (cIAP1)
Birc3 (cIAP2)
Cyld
Smac/ Diablo
Casp8 (Caspase 8)
B
Members of the NF-κB family
Tnfrsf1a-/-
Tnfrsf1a-/- IKKΔTCD4
Proteins with receptor, adaptor or kinase function
Inhibitors of cell death
Inducers of cell death
Cflar (c-FLIP)

Figure 6.3 At high concentrations, the Smac mimetic, birinapant induces TNF independent death among WT, SP thymocytes

Thymocytes from C57BL/6 wild-type mice were cultured for 16hr in medium containing either 200ng/mL TNF or PBS vehicle. The cells were then cultured for a further 5hr with either 100ng/mL TNF or PBS vehicle and with either the indicated concentration of the Smac mimetic birinapant or with DMSO vehicle. Following this, cells were analysed for viability. Duplicate wells were employed for each concentration of birinapant or DMSO. The culture was performed twice with similar results. Graphs show data from one of the two experiments. Mean % cell death ± SEM is shown.

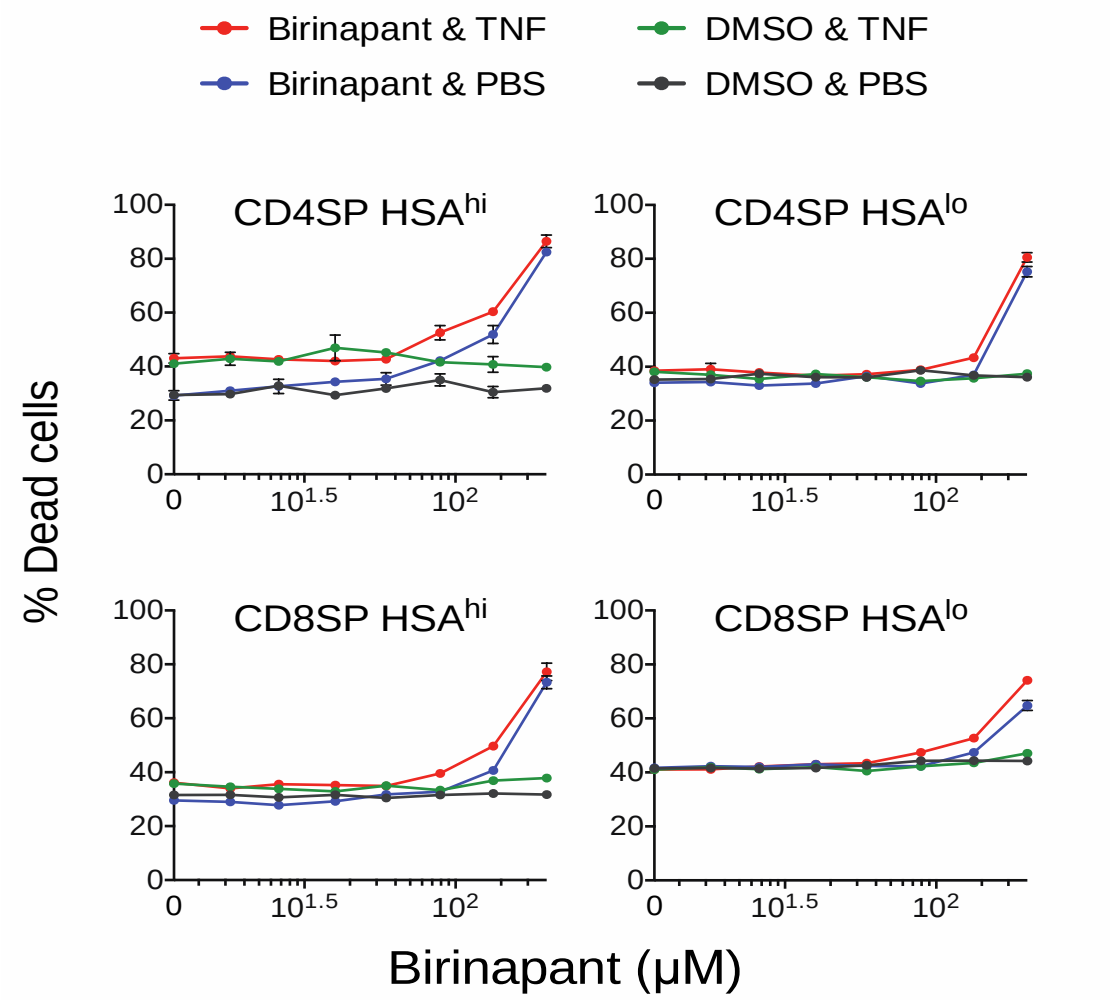

Birinapant & TNF
DMSO & TNF
Birinapant & PBS
DMSO & PBS
CD4SP HSA^hi
CD4SP HSA^lo
CD8SP HSA^hi
CD8SP HSA^lo
% Dead cells
Birinapant (μM)
100
80
60
40
20
0
0
10^1.5
10^2

Figure 6.4 A titration of TNFR1 blocking antibody reveals TNFR1 levels to be similar among different SP thymocyte populations

huCD2^{iCre} *Ikk1*$^{fx/fx}$ R26R^{EYFP} thymocytes were pre-incubated with a blocking antibody towards TNFR1 for 2 hours. Following this, the IKK1 deficient thymocytes received 10μM of the IKK2 inhibitor, BI605906, plus 30ng/mL TNF cytokine. The cells were then cultured for a further 24hr (the TNFR1 blocking Ab being present throughout). Technical replicates were employed by way of triplicate wells. Following culture, FACS analysis was used to assess the percentage of dead cells among each SP population. The straight, solid, horizontal lines represent the background level of cell death (the percentage of death in the presence of IKK2 inhibitor, but with H_2O vehicle as opposed to TNF). Nonlinear regression was performed. Dotted, vertical lines represent the IC_{50} values for the different SP subsets – i.e. the concentration of TNFR1 blocking antibody needed to reduce maximum TNF induced death by 50%. The IC_{50} values are also illustrated by way of a bar chart (below). IC_{50} values are as follows: CD8SP HSA^{lo}, 9.7μg/mL; CD8SP HSA^{hi}, 8.9μg/mL; CD4SP HSA^{hi}, 6.9μg/mL; CD4SP HSA^{lo}, 1.5μg/mL. Line graphs show mean ± SEM.

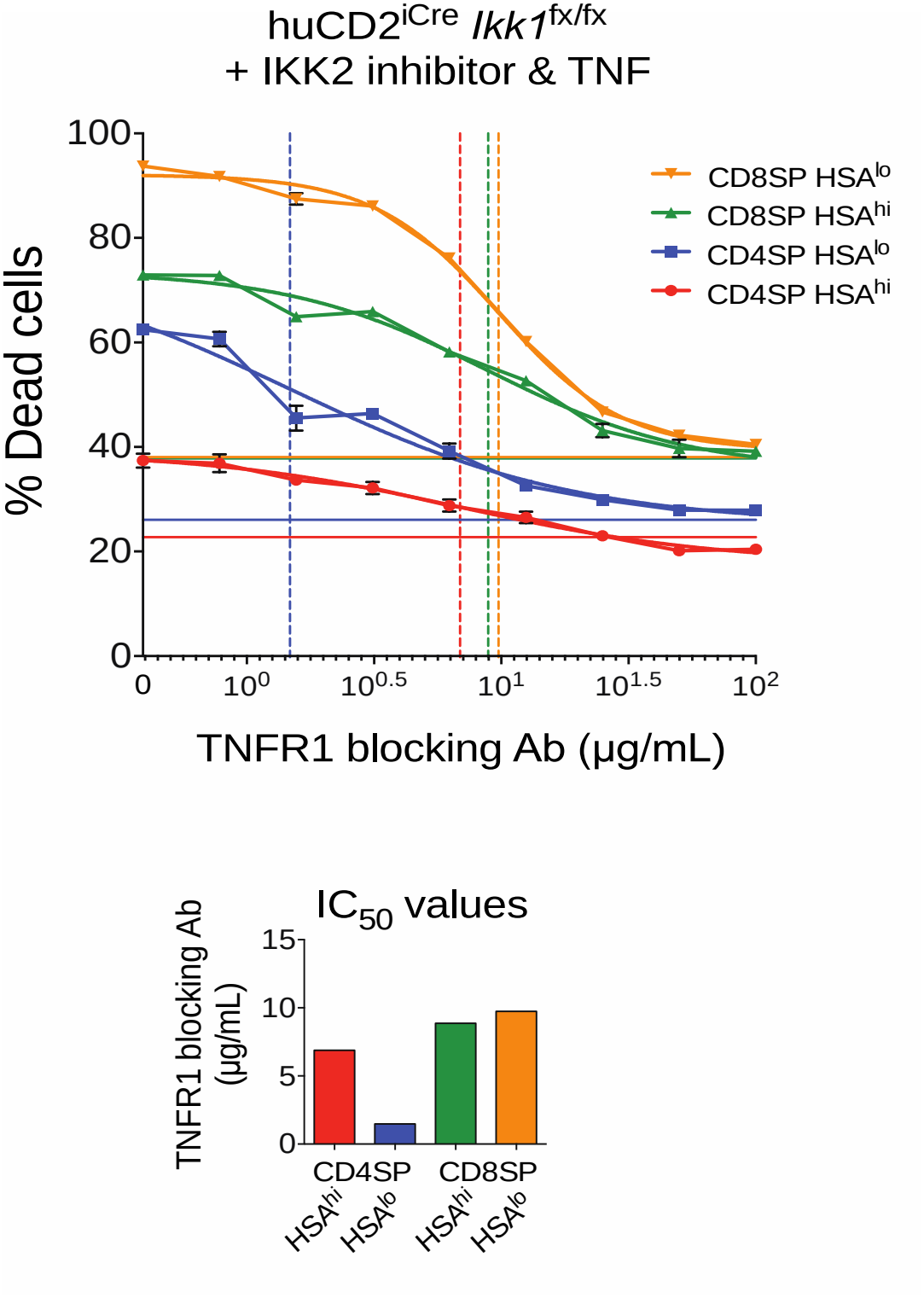

huCD2iCre Ikk1fx/fx
+ IKK2 inhibitor & TNF
% Dead cells
100
80
60
40
20
0
0
10^0
10^0.5
10^1
10^1.5
10^2
TNFR1 blocking Ab (µg/mL)
CD8SP HSAlo
CD8SP HSAhi
CD4SP HSAlo
CD4SP HSAhi
IC50 values
TNFR1 blocking Ab (µg/mL)
15
10
5
0
CD4SP HSAhi
CD4SP HSAlo
CD8SP HSAhi
CD8SP HSAlo

Figure 6.5 RIPK1 is developmentally regulated among thymocytes

Thymocytes from C57BL/6 (WT) and *Tnfrsf1a*$^{-/-}$ IKKΔT^{CD4} mice were analysed by FACS for their expression of RIPK1. (**A**) Density plots show the gating strategies employed. All density plots show the thymus of a WT mouse. Histograms show expression of RIPK1 protein within the DN, DP, and SP thymocyte populations of a WT mouse. (**B**) The bar chart to the left shows the mean fluorescence intensity (MFI) of RIPK1 protein for the DN, DP, and SP thymocyte populations of WT and *Tnfrsf1a*$^{-/-}$ IKKΔT^{CD4} mice. Each bar shows the average MFI of 2 mice. The experiment was performed twice with similar results. One representative experiment is shown. The bar chart to the right shows RNA sequencing data for the RIPK1 gene (*Ripk1*; data first shown in **Figure 6.1A**). Bars show mean ± SEM.

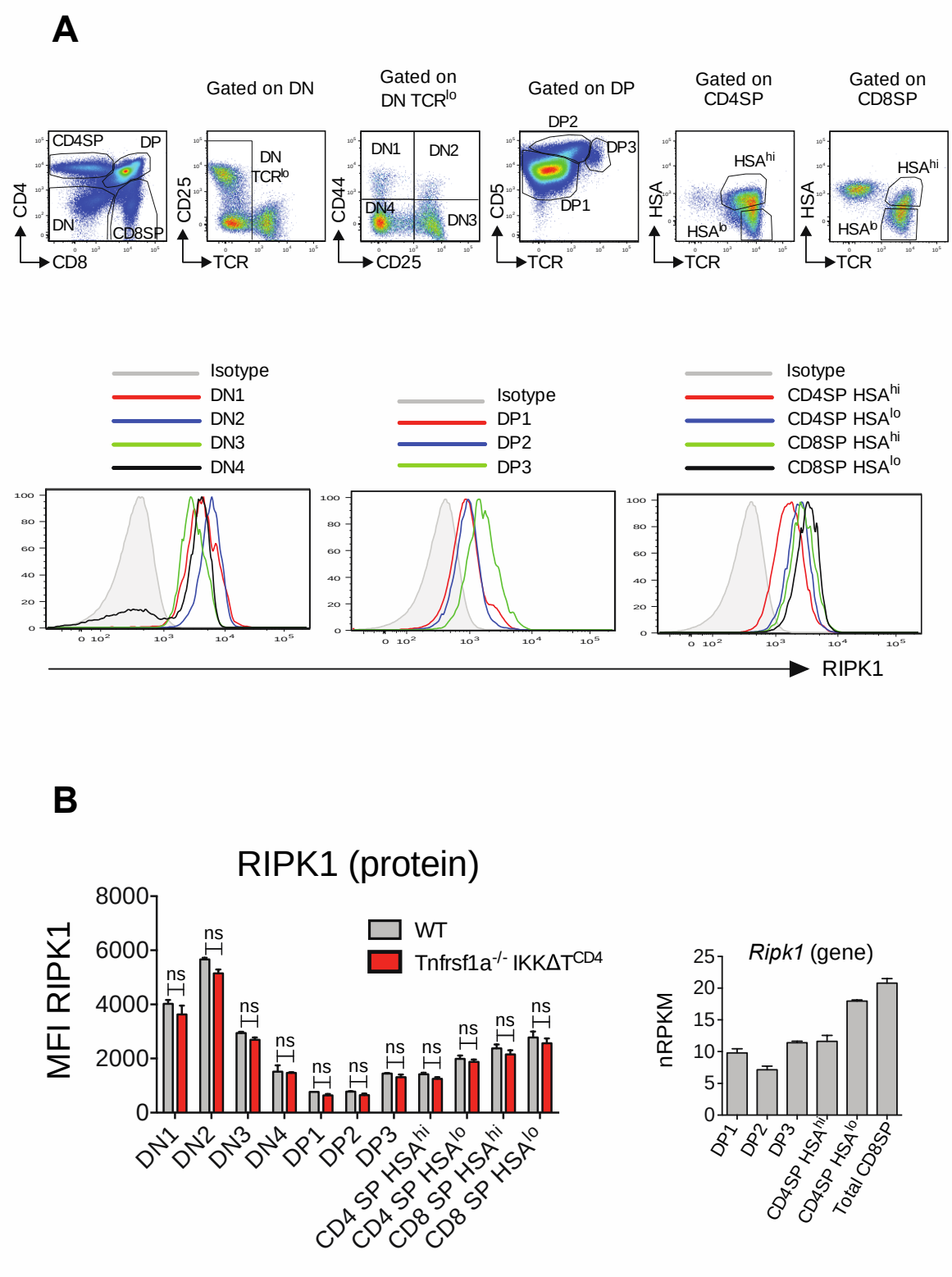

A
Gated on DN
Gated on DN TCRlo
Gated on DP
Gated on CD4SP
Gated on CD8SP
CD4SP
DP
DN
CD8SP
CD4
CD8
DN TCRlo
CD25
TCR
DN1
DN2
DN4
DN3
CD44
CD25
DP2
DP3
DP1
CD5
TCR
HSAhi
HSAlo
HSA
TCR
Isotype
DN1
DN2
DN3
DN4
Isotype
DP1
DP2
DP3
Isotype
CD4SP HSAhi
CD4SP HSAlo
CD8SP HSAhi
CD8SP HSAlo
RIPK1
B
RIPK1 (protein)
MFI RIPK1
WT
Tnfrsf1a-/- IKKΔTCD4
ns
DN1
DN2
DN3
DN4
DP1
DP2
DP3
CD4 SP HSAhi
CD4 SP HSAlo
CD8 SP HSAhi
CD8 SP HSAlo
Ripk1 (gene)
nRPKM
DP1
DP2
DP3
CD4SP HSAhi
CD4SP HSAlo
Total CD8SP

Figure 6.6 Inhibition of RIPK1 can protect IKK deficient SP thymocytes from TNF induced caspase-8 activation and death

Thymocytes from huCD2^{iCre} *Ikk1*$^{fx/fx}$ R26R^{EYFP} mice were pre-incubated with the RIPK1 inhibitor necrostatin-1. (**A & B**) Following a 2hr pre-incubation with necrostatin-1, the IKK1 deficient thymocytes received 10μM of the IKK2 inhibitor, BI605906, plus either 30ng/mL TNF cytokine or H_2O vehicle. (**A**) The cells were then cultured for a further 21hr (the indicated concentration of necrostatin-1 being present throughout). At the end of the culture period, cells were assessed for viability. The line graphs show the percentage of dead cells among each SP population. Nonlinear regression was performed. Dotted, vertical lines on the line graphs represent the IC_{50} values for the different SP subsets – i.e. the concentration of necrostatin-1 needed to reduce TNF induced death by 50%. The bar chart below also shows the IC_{50} values. The IC_{50} values are as follows: CD8SP HSA^{lo}, 2.8μM; CD4SP HSA^{lo}, 1.2μM; CD8SP HSA^{hi}, 0.9μM; CD4SP HSA^{hi}, 0.7μM. (**B**) Following addition of IKK2 inhibitor ±TNF, the cells were cultured for a further 4hr. Caspase-8 activation (within live cells) and total cell death were then measured among the CD8SP HSA^{lo} population. (**C**) Following a 5hr pre-incubation with necrostatin-1, the IKK1 deficient cells were cultured for a further 16hr either with or without the addition of TNF cytokine (no IKK2 inhibitor being present). The mean fluorescence intensity (MFI) of the IL-7R was then calculated by FACS for each of the SP populations.

Line graphs show mean ± SEM.

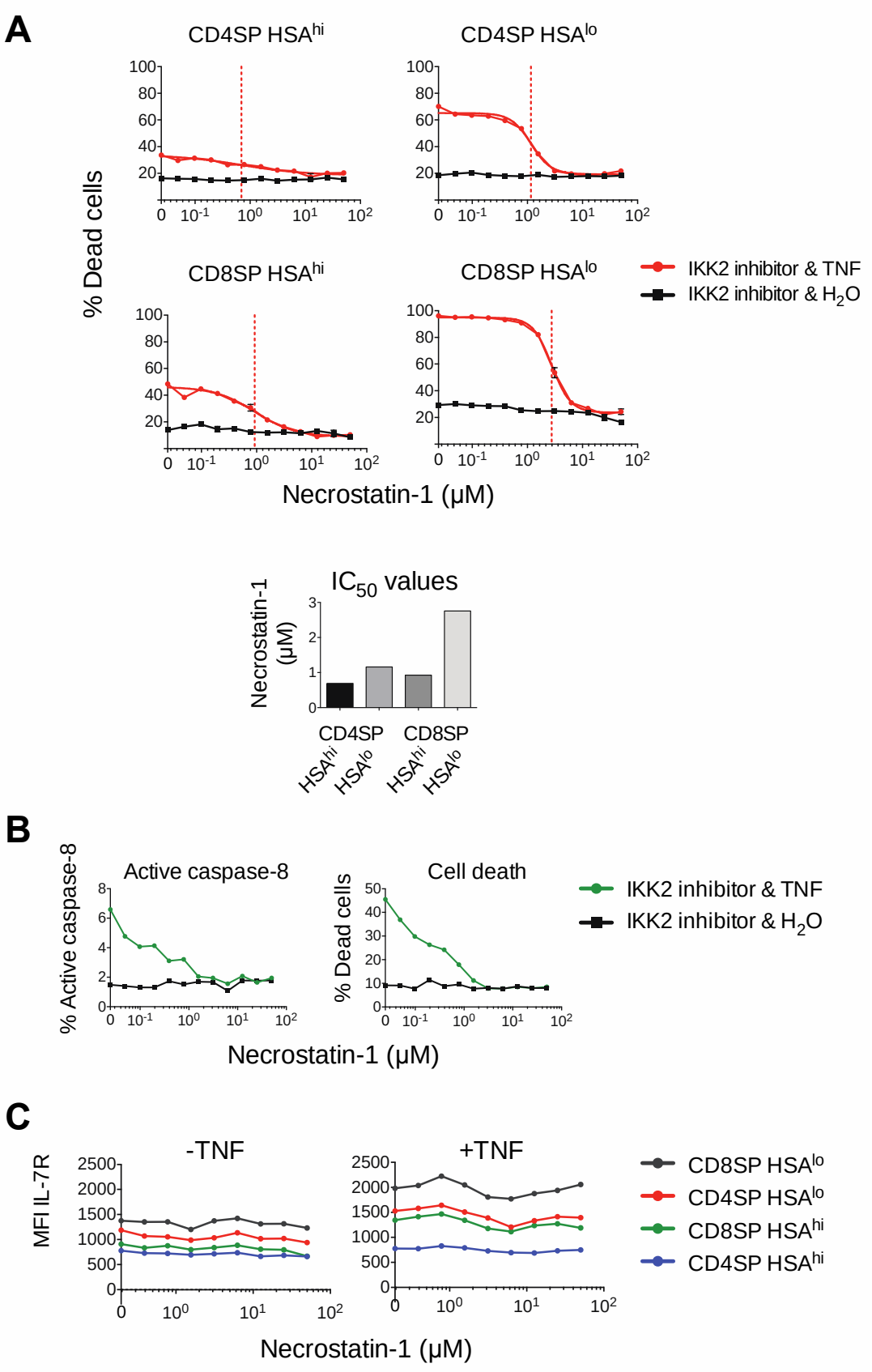
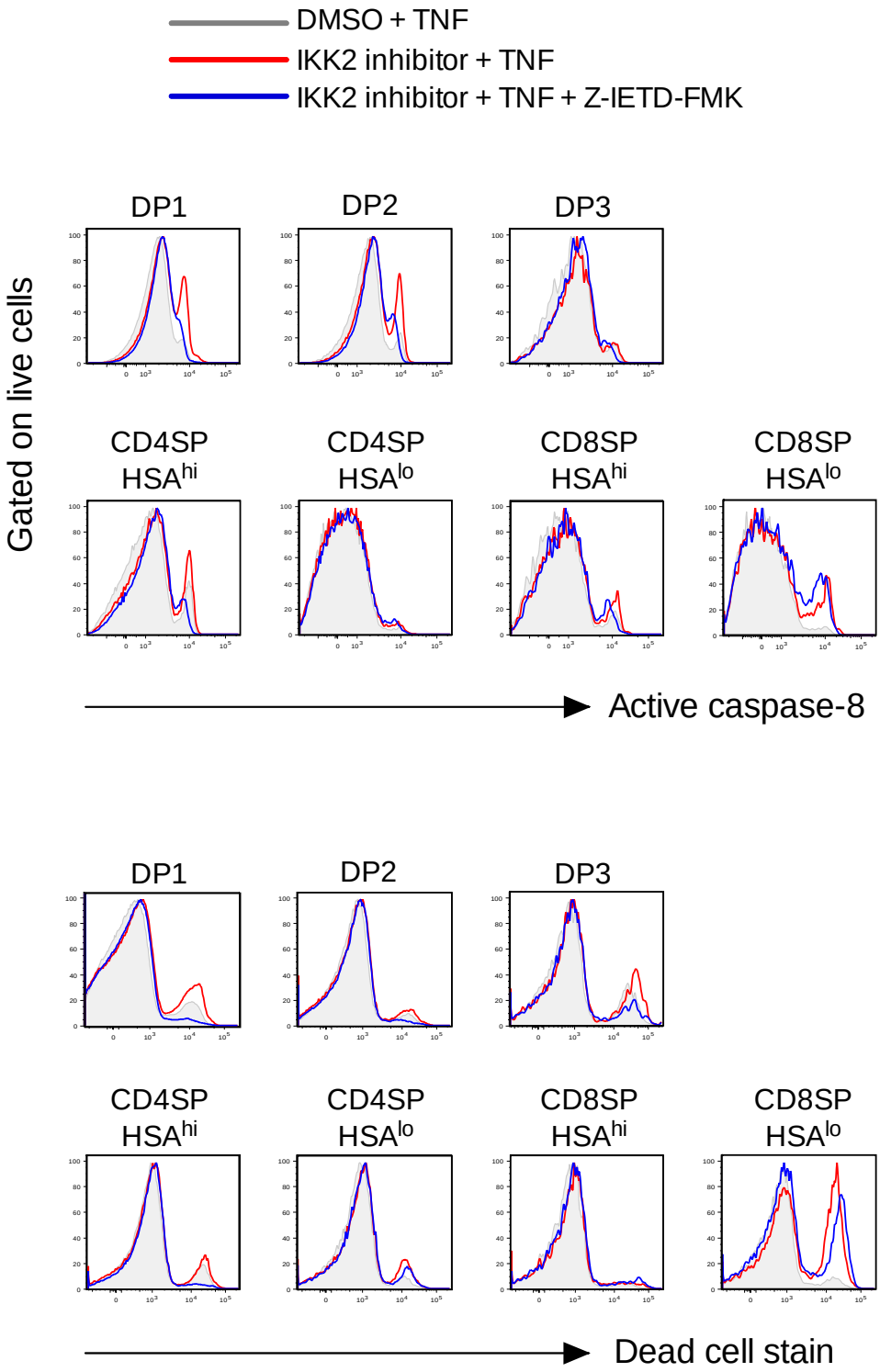

A
CD4SP HSAhi
CD4SP HSAlo
CD8SP HSAhi
CD8SP HSAlo
% Dead cells
IKK2 inhibitor & TNF
IKK2 inhibitor & H2O
Necrostatin-1 (μM)
IC50 values
Necrostatin-1 (μM)
CD4SP
CD8SP
HSAhi
HSAlo
B
Active caspase-8
Cell death
% Active caspase-8
% Dead cells
IKK2 inhibitor & TNF
IKK2 inhibitor & H2O
Necrostatin-1 (μM)
C
-TNF
+TNF
MFI IL-7R
CD8SP HSAlo
CD4SP HSAlo
CD8SP HSAhi
CD4SP HSAhi
Necrostatin-1 (μM)

Figure 6.7 In IKK deficient thymocytes, TNF dependent cell death appears to be largely caspase-8 dependent

Thymocytes from huCD2^{iCre} *Ikk1*$^{fx/fx}$ R26R^{EYFP} mice were pre-incubated for 1hr with either medium alone or medium containing the caspase-8 inhibitor, Z-IETD-FMK (40μM). The cells that had been pre-incubated in medium were then cultured for 4hr with TNF cytokine (30ng/mL) plus either DMSO vehicle, or the IKK2 inhibitor, BI605906 (10μM). Alternatively, cells that had been pre-incubated with Z-IETD-FMK were cultured for 4hr with TNF cytokine (30ng/mL), plus both BI605906 (10μM) and Z-IETD-FMK (20μM). At the end of the culture, the cells were stained for 1hr with Red-IETD-FMK, thus enabling quantification of any active caspase-8 present. Finally, cells were stained for surface markers and for viability. The top two rows of histograms show active caspase-8 staining within living cells of the indicated thymocyte populations. The bottom two rows of histograms show viability within the different thymocyte populations. As a control for Z-IETD-FMK, the negative control for caspase inhibitors, Z-FA-FMK, was added to some of the cells. This was found to have no inhibitory effect on either the percentage of active caspase-8 or on cell death (data not shown).


DMSO + TNF
IKK2 inhibitor + TNF
IKK2 inhibitor + TNF + Z-IETD-FMK
Gated on live cells
DP1
DP2
DP3
CD4SP HSAhi
CD4SP HSAlo
CD8SP HSAhi
CD8SP HSAlo
Active caspase-8
DP1
DP2
DP3
CD4SP HSAhi
CD4SP HSAlo
CD8SP HSAhi
CD8SP HSAlo
Dead cell stain

Schematic 6.1 -Chapter 6 summary- Activation of NF-κB, apoptosis, and necroptosis pathways by TNFR1

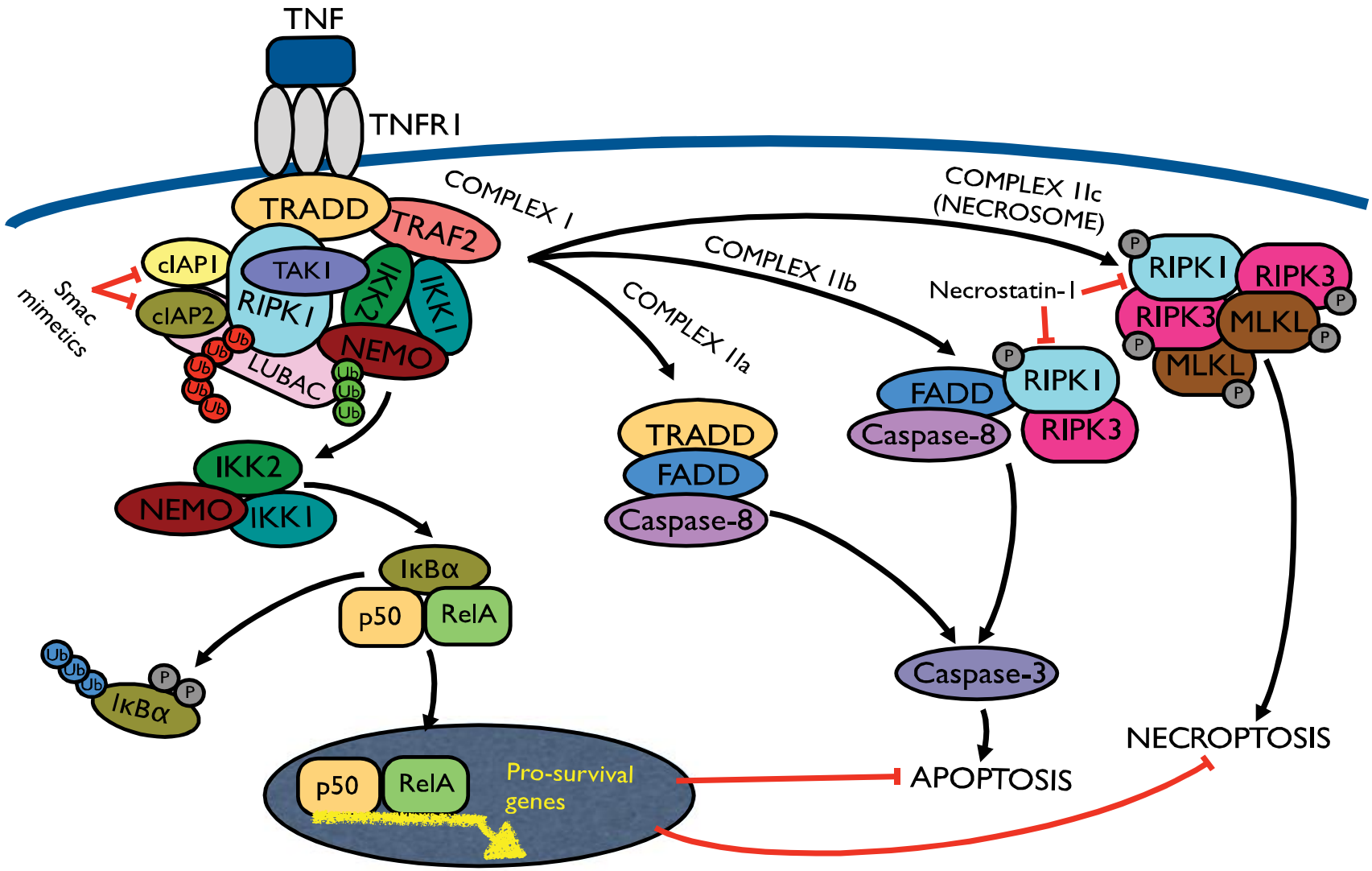
When TNF cytokine binds to TNFR1, conformation changes are induced on the receptor. This enables recruitment of TRADD to the death domain of TNFR1 (Hsu et al., 1995). In turn, TRADD recruits RIPK1 and TRAF2 (Hsu et al., 1996a; 1996b). TRAF2 interacts with IKK1 and IKK2 of the IKK complex (Devin et al., 2001), whilst RIPK1 interacts with NEMO (Zhang et al., 2000). RIPK1 also interacts with TAK1, thus bringing an IKK kinase into close proximity to the IKK complex (Ea et al., 2006). Evidence suggests that the kinase function of RIPK1 is not required for IKK recruitment (Lee et al., 2004). However, K63-linked ubiquitination of RIPK1 is thought necessary for the recruitment of both the IKK complex and TAK1 (Ea et al., 2006). cIAP1 and cIAP2 are believed to enable this K63-linked ubiquitination (red circles) of RIPK1 (Yin et al., 2009). Smac mimetics cause degradation of the cIAPs (Benetatos et al., 2014). The multi-protein structure formed around TNFR1 is called complex I. It may be stabilized by the LUBAC, which is itself recruited by the cIAPs (Haas et al., 2009; Kirisako et al., 2006). Evidence suggests that the LUBAC adds linear ubiquitin chains (green circles) onto NEMO, resulting in direct activation of the IKK complex (Fujita et al., 2014). Complex I mediated activation of the IKK complex results in phosphorylation of the IκBs, allowing for their K48-linked ubiquitination (blue circles) and degradation by the proteasome. This frees the NF-κB dimers allowing them to translocate to the nucleus. NF-κB activation results in the production of pro-survival proteins, necessary to counteract TNF induced cell death.

If complex I becomes destabilized, then a variety of cytosolic complexes may be formed. In complex IIa, TRADD binds to FADD, resulting in activation of caspase-8, which in turn leads to activation of caspase-3 and apoptosis. Complex IIb may be formed in situations where there is a deficiency of cIAPs, TAK1 or NEMO (Dondelinger et al., 2013; Legarda-Addison et al., 2009; Pasparakis and Vandenabeele, 2015; Wang et al., 2008). Complex IIb formation relies on the recruitment of RIPK3 to the cytosolic complex and results in RIPK1 mediated apoptosis. The kinase activity of RIPK1 is believed to

be essential for apoptosis via complex IIb (Conrad et al., 2016). Necrostatin-1 inhibits RIPK1 kinase activity (Degterev et al., 2008). Complex IIc, also called the necrosome, is formed in the absence of caspase-8 and the presence of high levels of RIPK3 and its substrate MLKL (Pasparakis and Vandenabeele, 2015). Formation of the necrosome leads to a kinase-regulated form of necrotic cell death called necroptosis. Necroptosis is believed to rely on RIPK1 kinase activity and hence to also be inhibited by necrostatin-1 (Berger et al., 2014; Ofengeim and Yuan, 2013).

The balance between the pro-survival NF-κB pathway and the pro-death pathways ultimately determines the fate of a TNF stimulated cell.

Figure drawn by myself. Figure partially inspired by (Conrad et al., 2016).


TNF
TNFR1
TRADD
TRAF2
cIAP1
cIAP2
TAK1
RIPK1
IKK2
IKK1
NEMO
LUBAC
Ub
Smac mimetics
COMPLEX I
COMPLEX IIa
COMPLEX IIb
COMPLEX IIc (NECROSOME)
Necrostatin-1
P
RIPK1
RIPK3
MLKL
FADD
Caspase-8
IKK2
NEMO
IKK1
IκBα
p50
RelA
Caspase-3
Pro-survival genes
APOPTOSIS
NECROPTOSIS

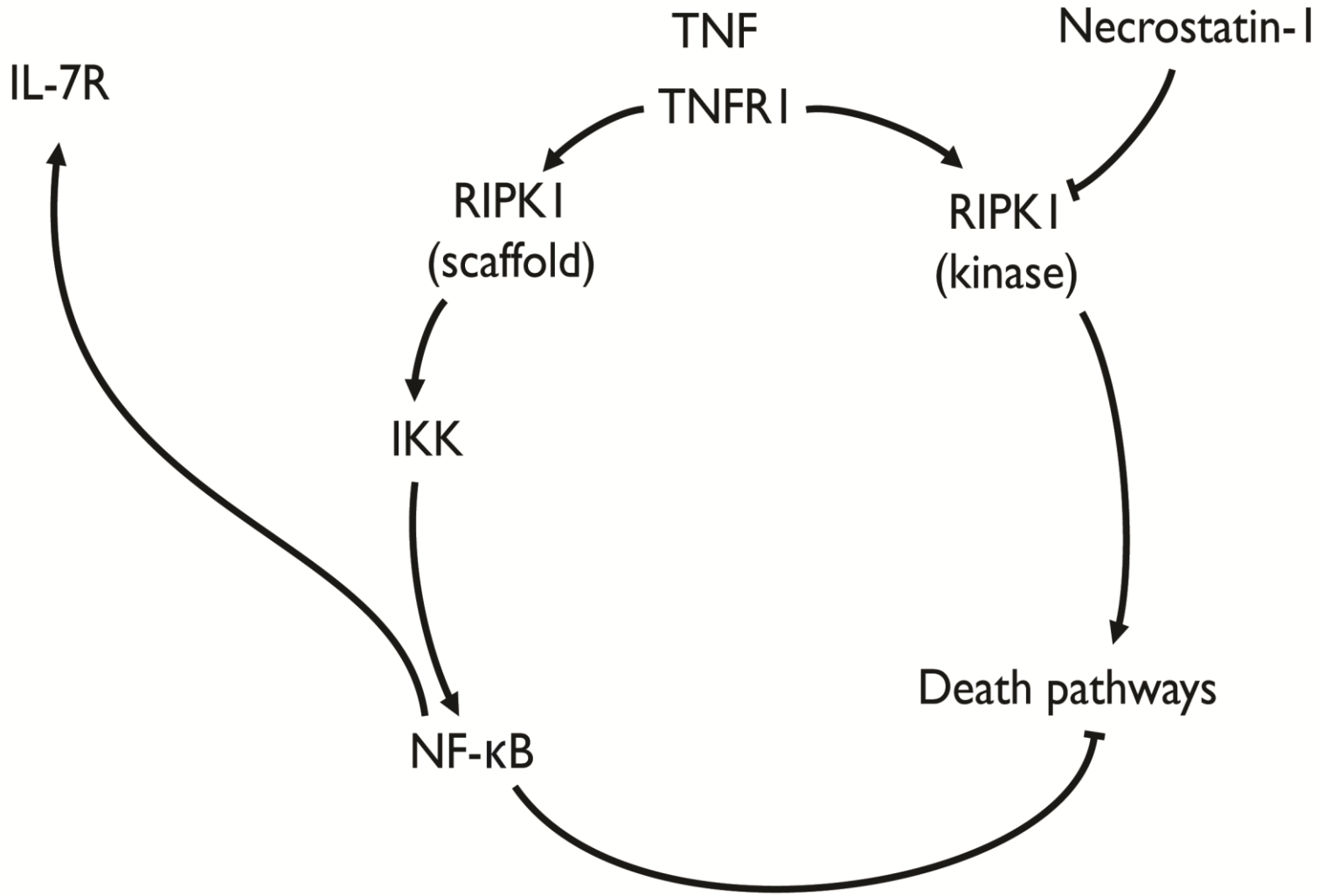
Chapter 7 Final Discussion

The aim of this thesis was to investigate the role of NF-κB signalling in thymocyte development and homeostasis. Whilst the importance of NF-κB signalling in T cell development has previously been appreciated, the nature of the activating stimuli, and the downstream signalling pathways linking them to NF-κB activation have remained elusive. We began by examining the phenotype of mice with a conditional deletion of both *Ikk1* and *Ikk2* early in thymocyte development. This allowed us to understand the block in T cell development that occurs in the absence of IKK mediated NF-κB signalling. We next attempted to identify the stimuli responsible for NF-κB signalling during normal thymopoiesis. Through an *in vivo* approach we noted that TNF induced death accounted for the thymic phenotype of the IKK deficient mice. Then, through an *in vitro* approach, we identified TNF as an activator of NF-κB regulated gene expression in SP thymocytes. Finally, we investigated the signalling pathway linking TNFR1 stimulation to activation of NF-κB. We identified RIPK1 as the mediator of TNF induced death and also found strong correlations between RIPK1 abundance and upregulation of NF-κB dependent IL-7R expression. In addition, we identified the cIAPs as key targets of TNF induced NF-κB signalling that likely function to inhibit TNF induced death of SP thymocytes.

The data presented in this thesis supports a previously unidentified role for NF-κB signalling during the SP stage of thymocyte development, where it is required to counteract RIPK1 mediated, TNF induced apoptosis. The data presented here also supports earlier studies that show NF-κB signalling to be necessary for upregulation of the IL-7R during the late stages of thymocyte development (Silva et al., 2014). In the NF-κB dependent upregulation of IL-7R expression, we and others have identified TNF as an activating stimulus (Silva et al., 2014). However, we found TNF to play a redundant role, possibly with other TNFRSF members, in the induction of the IL-7R.

Schematic 7.1 -Final summary- RIPK1 is an important mediator between TNF induced death and TNF induced survival

NF-κB signalling is required during SP thymocyte development in order to protect against TNF induced death and to enable IL-7R upregulation as cells leave the thymus. In SP thymocytes, TNF itself is an activator of NF-κB induced IL-7R upregulation. RIPK1 is a cruicial mediator between TNF induced activation of pro-death pathways and TNF induced activation of NF-κB. RIPK1 kinase activity is required for the activation of certain pro-death pathways, but not for NF-κB activation and IL-7R upregulation. Hence, the RIPK1 kinase inhibitor, necrostatin-1, can prevent the TNF induced death of NF-κB deficient thymocytes, while having no effect on IL-7R expression. RIPK1 can also function as a scaffold, enabling IKK recruitment and activation downstream of TNFR1.


TNF
Necrostatin-1
IL-7R
TNFR1
RIPK1
(scaffold)
RIPK1
(kinase)
IKK
Death pathways
NF-κB

7.1 NF-κB signalling prevents TNF induced apoptosis of SP thymocytes

Although IKK gene deletion occurred early during thymopoiesis in IKKΔT strains, T cell development remained unperturbed until the late SP stage. *In vivo* blockade of TNF signalling revealed that, in the absence of IKK, the loss of SP thymocyte populations could be attributed to TNF induced death. Hence, NF-κB signalling is necessary to counteract TNF induced cell death during the SP stage of thymocyte development. Through use of a combined genetic and pharmacological approach, we were able to acutely block NF-κB signalling in SP thymocytes *in vitro*. Importantly, this indicated that NF-κB plays a very active, as opposed to developmental, role in protecting SP thymocytes from TNF induced death. Rather than being required at discrete stages during T cell development in order to produce a population of cells resistant to TNF, NF-κB signalling was actively required during the SP stage for continued protection against TNF. NF-κB signalling is known to induce transcription of a number of anti-apoptotic genes. However, among SP thymocytes, we found that TNF induced NF-κB activation led to particularly high expression of the genes for cIAP1 and cIAP2. We believe that NF-κB induced expression of cIAPs protects TNF responsive SP thymocyte subsets from TNF induced death.

7.2 TNF is an activator of NF-κB in SP thymocytes

Whilst blocking TNF signalling in IKKΔT^{CD4} mice identified a clear role for NF-κB in ensuring SP survival, it alone did not identify the activator of NF-κB signalling. It is well established that TNF binding to TNFR1 induces activation of two distinct signalling pathways, one leading to complex I formation and NF-κB activation, and the other resulting in formation of complex II and promoting cell death. Since TNF was known to be a strong NF-κB activator, we hypothesised that TNF itself could be responsible for activating NF-κB during the SP stage, as well as for inducing cell death in the absence of IKK. Our hypothesis was confirmed by cell culture experiments. From the phenotype of the IKK deficient and Rel family member deficient mice that we examined and from earlier

studies, we knew IL-7R expression to be a reliable readout of the strength of NF-κB activity (Miller et al., 2014; Silva et al., 2014). When WT thymocytes were cultured with TNF, the SP subsets were able to upregulate their expression of IL-7R. IKK deficient thymocytes were, however, unable to increase IL-7R expression upon culture with TNF. A similar result has previously been shown with F5 *Rag1*$^{-/-}$ CD8SP thymocytes, which, when deficient in IKK2, were limited in their capacity to upregulate IL-7R in response to TNF (Silva et al., 2014). From our *in vivo* and *in vitro* studies we therefore concluded that SP thymocytes are responsive to TNF signalling. In wild-type, SP cells it seems that the NF-κB pathway activated by TNF works to balance the apoptotic pathway that is simultaneously induced by TNF, so that cell survival results. Ultimately, TNF was shown to be an activator of NF-κB during the SP stage of thymocyte development.

7.3 TNFR ligation, and not TCR ligation, is the stimulus for NF-κB activation during the SP stage

Previous studies have suggested that TCR signalling activates NF-κB in SP thymocytes. A lack of TCR mediated NF-κB activation was deemed responsible for the loss of HSA^{lo} SP thymocytes in TAK1 deficient mice, although no evidence for this was provided (Wan et al., 2006). We propose that TNFR ligation, as opposed to TCR ligation, is the stimulus for NF-κB activation during the SP stage of T cell development. In support of our claim, mice which lack TCR signalling during the SP stage of thymocyte development do not phenocopy the TAK1 deficient mice (Sinclair and Seddon, 2014).

Notably, the IKKΔT mice phenocopied mice with a CD4^{Cre} mediated deficiency of NEMO (Schmidt-Supprian et al., 2003) and mice with an Lck^{Cre} mediated deficiency of TAK1 (Liu et al., 2006; Sato et al., 2006). Hence, despite use of Cre recombinases that delete at the DP stage of thymocyte development or earlier, the block in T cell development in mice lacking NF-κB signalling only occurs between the HSA^{hi} and HSA^{lo} SP stages. This suggests that NF-κB signalling is dispensable downstream of the pre-TCR during beta-selection and

of the TCR during the positive and negative selection stages. In mice expressing a super-inhibitory form of IκBα, CD8SP thymocytes are reduced in number. This has previously been attributed to poor positive selection in the absence of TCR mediated NF-κB signalling (Esslinger et al., 1997; Hettmann and Leiden, 2000; Jimi et al., 2008; Mora et al., 1999). However, through examination of super-inhibitor expressing pLck-IκB-PEST mice, we found that the reduction in CD8SP numbers could be attributed to loss of the HSA^{lo} subset. The fact that the HSA^{hi} subset remained unaffected suggested that normal positive selection had taken place. Furthermore, we found that the reduction in CD8SP HSA^{lo} thymocytes in pLck-IκB-PEST mice was solely due to the TNF induced death of this population. Again such data suggests that NF-κB signalling is not required during the DP stage of thymocyte development, but rather is required during the SP stage to balance TNF induced death signals.

7.4 NF-κB signalling is required for correct differentiation of SP thymocytes

Haematopoietic progenitors within the fetal liver produce large quantities of TNF (Rosenfeld et al., 2000). This leads to hepatocyte death and embryonic lethality in IKK2, NEMO, or RelA knockout mice (Beg et al., 1995; Li et al., 1999b; 1999c; Rudolph et al., 2000). However, the embryonic lethal phenotypes of IKK2 and RelA knockout mice can be reversed by inactivation of the genes for TNFR1 or TNF (Doi et al., 1999; Li et al., 1999b; Rosenfeld et al., 2000; Senftleben et al., 2001b). Such experiments have demonstrated that NF-κB signalling is not required for the actual development/differentiation of liver cells, but rather for their protection from death (Karin and Lin, 2002).

The fact that IKK deficient SP thymocytes could be rescued by blocking TNF signalling *in vivo* suggested that, as with hepatocytes, NF-κB signalling was not required for SP differentiation *per se*. However, the SP cells that developed in the absence of IKK were found to be functionally deficient in IL-7R signalling. Although the *in vitro* addition of IL-7 cytokine was able to significantly reduce death among wild-type, SP thymocytes, it had limited capacity to promote the

survival of SP thymocytes from IKK deficient mice. Furthermore, we and others have shown that the naïve T cells in the periphery of mice with a block in NF-κB signalling have reduced expression of the IL-7R (Silva et al., 2014). Hence, it seems that NF-κB signalling is required during the SP stage to enable generation of a naïve T cell pool that is functionally responsive to low concentrations of IL-7 cytokine. Of note, maturation of SP thymocytes and their emigration into the periphery does not require IL-7 signalling (Weinreich et al., 2011). However, the long-term survival of naïve T cells in the periphery is dependent on IL-7R expression (Schluns et al., 2000). Therefore, NF-κB induced IL-7R expression during the SP stage is necessary for normal T cell homeostasis in the periphery. We conclude that, in addition to a survival function, NF-κB signalling during the SP stage of development does play a role in T cell differentiation.

7.5 Increasing RIPK1 expression as SP thymocytes mature has a functional significance

Through *in vitro* and *in vivo* experiments we found that different thymic subsets have distinct susceptibilities to TNF induced death. In the absence of IKK signalling, the DP and CD4SP HSA^{hi} subsets were no more susceptible to TNF induced death than their IKK sufficient counterparts. However, upon loss of IKK signalling, CD4SP HSA^{lo}, CD8SP HSA^{hi}, and CD8SP HSA^{lo} subsets showed hugely increased susceptibilities towards TNF.

TNFR1 expression could not be detected by flow cytometry. Therefore, we indirectly measured TNFR1 expression on SP thymocytes by biological inhibition of TNF induced death with an anti-TNFR1 blocking mAb. This revealed that all SP subsets had largely similar expression of TNFR1. Hence, the distinct susceptibilities of SP subsets towards TNF could not be explained by differences in TNFR1 expression. Through use of RNA sequencing, flow cytometry, and the RIPK1 inhibitor, necrostatin-1, we found that SP subsets differed in their expression of RIPK1. RIPK1 is a mediator of the life versus death decisions a cell makes in response to TNF, being involved in activation of

both complex I and complex II downstream of TNFR1 (Christofferson et al., 2014; Festjens et al., 2007). In the absence of IKK signalling, the abundance of RIPK1 within the thymocyte population was found to determine its susceptibility towards TNF induced death. IKK deficient CD8SP HSA^{lo} cells had the highest expression of RIPK1 and showed the greatest sensitivity towards TNF. In comparison, IKK deficient DP and CD4SP HSA^{hi} cells expressed only low amounts of RIPK1 and consequently, showed great resistance to TNF. Blocking the kinase activity of RIPK1, through use of necrostatin-1, completely rescued IKK deficient SP populations from TNF induced death. Hence, TNF induced death was found to be entirely RIPK1 dependent.

In wild-type cells, RIPK1 abundance did not determine susceptibility towards TNF induced death, but rather was found to correlate with the extent of IL-7R upregulation in response to TNF. Upon *in vitro* stimulation with TNF, wild-type, CD4SP HSA^{hi} cells, which contained little RIPK1, showed barely any IL-7R upregulation, whilst CD8SP cells substantially increased their IL-7R expression. The production of CD8SP thymocytes is slower than that of CD4SP thymocytes (Saini et al., 2010), and HSA^{lo} cells are more mature than HSA^{hi} cells (Tian et al., 2001). Ultimately then, it seems that, until SP thymocytes reach a certain level of maturity, they remain unresponsive to TNF signalling. We propose that increasing RIPK1 expression, as cells progress through the SP stage of development, allows TNFR1 mediated NF-κB activation, which results in induction of *Il7r*.

Past studies have proposed that NF-κB signalling is more important for the development of the CD8 than the CD4 lineage of T cells (Hettmann and Leiden, 2000; Jimi et al., 2008; Mora et al., 1999). Furthermore, CD8SP thymocytes have been shown to have more transcriptionally active NF-κB than CD4SP thymocytes (Voll et al., 2000). We speculate that, during the SP stage of development, the abundance of RIPK1 may dictate the strength of signalling downstream of TNFR1 and hence, the extent of NF-κB activation. This could explain the greater NF-κB activity among the CD8 than the CD4 lineage.

7.6 Functional redundancy among TNFSF members

By enabling upregulation of the IL-7R in the final stages of SP development, TNF signalling via TNFR1 does appear to play a very functional role in the thymus. Importantly, however, mice deficient in TNFR1 have normal IL-7R expression. The SP thymocytes of *Tnfrsf1a*$^{-/-}$ mice are functionally responsive to IL-7R stimulation, and the naïve, peripheral T cells have normal IL-7R expression and are present in wild-type numbers. We and others found that CD27L, a member of the TNFSF of cytokines, was able to increase IL-7R expression on F5 *Rag1*$^{-/-}$ CD8SP thymocytes (Silva et al., 2014). As with TNF induced IL-7R upregulation, CD27L induced IL-7R upregulation was found to be dependent on the NF-κB signalling pathway (Silva et al., 2014). A huge amount of biological redundancy is known to exist among members of the TNFSF, all of which can activate NF-κB (Aggarwal et al., 2012; Mahmud et al., 2014). Thus, it is likely that a lack of TNFR1 induced NF-κB activation could be compensated for by signalling initiated by other receptors of the TNFSF. It is possible that a double deficiency of TNFR1 and CD27 may be adequate to cause a reduction in IL-7R expression, although a combined deficiency of several TNFSF ligands/receptors may be necessary. The medullary epithelial cells of the thymus are constitutive producers of TNF (Mahmud et al., 2014). SP thymocytes reside in the medulla of the thymus for an estimated 4 days, whilst undergoing the final stages of maturation (Sinclair et al., 2011). Therefore, it is likely that thymocytes are subject to much TNF signalling during the final stages of T cell maturation, and this is likely of functional significance.

Of note, signalling initiated by TNFSF members is required for the production of thymic T_{reg} cells (Coquet et al., 2013; Mahmud et al., 2014). T_{reg} cell development is believed to require strong positive selection signalling as initiated by the TCR. In response to TCR signalling, T_{reg} cells upregulate their expression of GITR, OX40, and TNFR2, and the combined inhibition of all three receptors prevents T_{reg} development (Mahmud et al., 2014). Furthermore, signalling via CD27 is required for inhibition of the mitochondrial apoptosis pathway during the positive selection of T_{reg} cells (Coquet et al., 2013). Notably,

mice deficient in CD27 or its ligand have reduced thymic and peripheral T_{reg} cells (Coquet et al., 2013). Hence, in addition to upregulating IL-7R expression on SP thymocytes, TNFSF members have a clear role to play in T_{reg} cell generation.

We speculate that it is the activation of NF-κB by TNFSF members that is required for T_{reg} survival. In support of this, we found that even an incomplete block in NF-κB signalling, caused by a deficiency of either IKK1 or IKK2, significantly decreased thymic and peripheral T_{reg} numbers. It is possible that T_{reg} cells, developing in the thymus, are particularly reliant on the anti-apoptotic proteins induced by NF-κB signalling. Treatment of IKKΔT^{CD4} mice with anti-TNF mAb does not rescue T_{reg} numbers. Hence, TNF induced apoptosis is not responsible for the lack of T_{reg} cells in IKK deficient mice. However, as previously shown, the mitochondrial apoptosis pathway does cause death of T_{reg} cells (Coquet et al., 2013), and it is possible that CD27 mediated inhibition of this pathway works via NF-κB signalling.

7.7 The role of NF-κB signalling in peripheral T cell homeostasis

Blocking TNF signalling in IKKΔT^{CD4} mice, despite causing a near complete restoration of the SP thymic compartment, only partially restored naïve T cell numbers in the periphery. This suggested that the lifespan of IKK deficient, naïve T cells was reduced as compared to their wild-type counterparts. The low level of IL-7R expression on the IKK deficient, naïve T cells would certainly inhibit their long term survival (Schluns et al., 2000). Yet, as to whether the lack of peripheral, naïve T cells in *Tnfrsf1a*$^{-/-}$ IKKΔT^{CD4} mice can be fully accounted for by the loss of IL-7R expression, remains to be determined. It is possible that NF-κB signalling is required in naïve T cells for their homeostatic maintenance.

Blocking the NF-κB signalling pathway results in a reduction in both memory and T_{reg} cells in the periphery. The reduction of peripheral T_{reg} cells can be at least partially accounted for by the loss of natural T_{reg} production in the thymus.

However, it is likely that induced T_{reg} cells are also absent or reduced. The reason for the loss of memory cells in IKK deficient and RelA/NF-κB1 double deficient mice is unknown. Notably, IL-7R signalling is required for the generation of $CD8^+$ memory T cells (Schluns et al., 2000). Hence, it is possible that low IL-7R expression on naïve T cells (as seen in IKK deficient mice) could hinder their differentiation into memory cells. However, it is likely that NF-κB signalling is also required among peripheral T cells. The production of memory and regulatory T cell populations requires TCR and co-stimulatory signalling. Such signalling is known to activate NF-κB (Gerondakis et al., 2013; Watts, 2004). However, as to how dependent the generation/maintenance of memory and T_{reg} cells is upon NF-κB signalling remains an important question to address in future studies.

7.8 Why do SP thymocytes express TNFR1?

Many TNFSF cytokines are produced by the dendritic cells and medullary epithelial cells within the thymic medulla (Coquet et al., 2013; Mahmud et al., 2014). Since all TNFSF members can activate NF-κB, albeit some only weakly, it seems almost counter-intuitive that TNFR1, a death domain containing receptor, should be expressed upon SP thymocytes. Why would the SP thymocyte risk expression of a receptor that could ultimately signal its demise? Especially when it seems that signalling via other receptors, for example CD27, can lead to similar functions, such as T_{reg} production and IL-7R expression. Perhaps the answer to this lies in the fact that many caspases, activated by death receptors, have conserved evolutionary roles, not only in cell death, but also in inflammation (Siegel, 2006).

In drosophilia, DREDD is the homologue to mammalian caspase-8. Interestingly, DREDD does not often cause apoptosis, but rather leads to activation of NF-κB (Siegel, 2006). DREDD cleaves Relish (the homologue of p105 and p100), allowing its entry into the nucleus (Stoven et al., 2003). In mammals, caspase-8 is not thought to activate NF-κB directly. However, evidence suggests that caspase-8 may activate NF-κB via binding to the CBM

(CARMA1/Bcl-10/MALT1) complex downstream of the TCR (Su et al., 2005). In fact, caspase-8 deficient Jurkat T cells show defective activation of NF-κB (Su et al., 2005). Hence it is possible that, upon TNFR1 stimulation, formation of complex II could work, via caspase-8, to activate NF-κB signalling. Although not essential for thymocyte development (Salmena et al., 2003), caspase-8 may well facilitate NF-κB activation within thymocytes, possibly in response to TCR stimulation.

7.9 Future studies

Within SP thymocytes, RNA sequencing identified the cIAPs as TNF induced and NF-κB dependent. It would be interesting to determine whether forced expression of the cIAPs could rescue IKK deficient SP thymocytes from TNF induced death. Of note, mice with a T cell specific conditional deletion of c-FLIP seem to phenocopy IKK deficient mice. In the c-FLIP deficient animals, T cell development is also arrested at the HSA^{lo} SP stage, and there are few peripheral T cells (Zhang and He, 2005). For this reason, c-FLIP may also be an important NF-κB target in SP thymocytes, although not identified as such in our study.

The role that RIPK1 plays in thymocytes requires further investigation. Our results have clearly indicated that RIPK1 kinase activity is required for TNF induced death, but not for NF-κB activation. However, whether or not the scaffold function of RIPK1 is essential for NF-κB activation remains controversial (Devin et al., 2001; Kelliher et al., 1998; Wong et al., 2010). Future work could investigate the thymic and peripheral phenotype of mice with a T cell specific deletion of RIPK1. Assuming RIPK1 is essential for NF-κB activation, we predict the naïve T cells of RIPK1 deficient mice to show reduced IL-7R expression.

The results of our study suggest that NF-κB signalling is necessary for peripheral T cell homeostasis. A tamoxifen inducible Cre recombinase could be used to delete IKK1 and IKK2 among fully developed T cells in the periphery.

This would separate the requirement for NF-κB during T cell development from its requirement among peripheral T cells. The role that NF-κB signalling plays in T cells during an immune response is also an important area for future investigation. Caspase-8 is required for T cell proliferation and activation (Siegel, 2006). Hence caspase-8 is highly involved in both non-apoptotic as well as pro-apoptotic pathways. Precisely how caspases are stimulated to induce differentiation/survival programmes as opposed to cell death is unknown. However, we predict that it involves the activation of NF-κB. Caspase-8 appears to co-operate with FADD and c-FLIP in the induction of T cell activation (Siegel, 2006). Both FADD deficient and caspase-8 deficient T cells show reduced expansion upon activation via the TCR (Salmena et al., 2003; Zhang et al., 2005). During T cell activation, death receptors, such as Fas, are likely to be stimulated and lead to caspase-8 activation. In a wild-type T cell it is likely that caspase-8 could contribute to NF-κB activation, resulting in progression of the T cell through the cell cycle. However, if NF-κB were to be inhibited, then it is possible that caspase-8 would instead lead to activation-induced death. Of note, RIPK1, caspase-8, or FADD deficient Jurkat T cells are impaired in their activation of NF-κB (Kreuz et al., 2004). Future studies should perhaps aim to address the relative contributions of caspase-8, RIPK1, FADD, c-FLIP, and NF-κB to the T cell proliferation versus activation-induced death programmes.

7.10 Clinical perspectives

Dysregulation of the NF-κB pathway is responsible for a huge number of diseases in animals and humans. These include chronic inflammatory diseases, immunodeficiency, and cancer. In certain cancers, TNF induced overactivation of NF-κB can lead to increased production of the cIAPs. Smac mimetics, such as birinapant, are emerging as novel agents to block cIAP mediated pro-survival signalling in cancer cells (Benetatos et al., 2014). Notably, use of Smac mimetics and TNF in combination results in increased apoptosis of cell lines (Wu et al., 2007). Different cell types are likely to induce distinct anti-apoptotic genes upon NF-κB signalling. Understanding which anti-apoptotic proteins are

vital to which cell types could lead to production of more targeted drugs for cancer treatment.

X-linked anhidrotic ectodermal dysplasia with immunodeficiency (XL-EDA-ID) is a disease that affects male patients. It occurs when mutations in the gene for NEMO inhibit, but do not completely prevent NF-κB activation and is characterized by a severe immunodeficiency (Courtois and Gilmore, 2006). In one particular reported case of XL-EDA-ID, the reduced expression of NEMO was caused by duplication of part of the NEMO gene. Occasionally somatic reversion of this mutation occurred among cells, thus enabling restoration of the wild-type NEMO sequence (Nishikomori et al., 2004). As a consequence of this somatic reversion, some T cells from this patient showed reduced NEMO expression, whilst others had normal expression levels (Nishikomori et al., 2004). Analysis of peripheral blood mononuclear cells (PBMCs) from the patient revealed particularly low numbers of $CD4^+$ T cells. Moreover, the majority of T cells present in the blood (both CD4 and CD8 lineages) were found to have normal expression of NEMO, indicating preferential survival of cells that had reverted to the normal NEMO sequence (Nishikomori et al., 2004). Interestingly, there were very few naïve phenotype T cells in the patient's blood, most T cells presenting instead with memory markers (Nishikomori et al., 2004).

Ultimately, the T cell defects observed in this XL-EDA-ID patient do seem to mirror those seen in our $IKK\Delta T^{CD4}$ mice. Given the findings of our study, we predict that the loss of T cells in the XL-EDA-ID patient is due to TNF induced death of SP thymocytes. Anti-TNF treatment would be likely to increase naïve T cell numbers in the XL-EDA-ID patient, although it would be unable to restore NF-κB functional activity. Long-term treatment of XL-EDA-ID patients with anti-TNF may not be hugely beneficial. However, a short course of anti-TNF blocking Ab treatment may be useful in certain situations. If given prior to the administration of childhood vaccines, for example, anti-TNF could increase the number of naïve T cells available to respond to the antigen. Other forms of EDA-ID have been reported. These include autosomal dominant forms that are due to mutations in NF-κB signalling components other than NEMO, for

example the IκB proteins. Careful monitoring of naïve T cell numbers in such patients may highlight situations when anti-TNF therapy could be beneficial.

This study has increased our understanding of the role that NF-κB signalling plays in T cell development. Ultimately, we hope that this may assist with future drug design and treatment plans for a range of human disorders.

References

Acuto, O., and Michel, F. (2003). CD28-mediated co-stimulation: a quantitative support for TCR signalling. Nat Rev Immunol *3*, 939–951.

Aderem, A., and Underhill, D.M. (1999). Mechanisms of phagocytosis in macrophages. Annu. Rev. Immunol. *17*, 593–623.

Aggarwal, B.B. (2003). Signalling pathways of the TNF superfamily: a double-edged sword. Nat Rev Immunol *3*, 745–756.

Aggarwal, B.B., Gupta, S.C., and Kim, J.H. (2012). Historical perspectives on tumor necrosis factor and its superfamily: 25 years later, a golden journey. Blood *119*, 651–665.

Aifantis, I., Gounari, F., Scorrano, L., Borowski, C., and Boehmer, von, H. (2001). Constitutive pre-TCR signaling promotes differentiation through Ca2+ mobilization and activation of NF-|[kappa]|B and NFAT. Nat Immunol *2*, 403–409.

Alcamo, E., Mizgerd, J.P., Horwitz, B.H., Bronson, R., Beg, A.A., Scott, M., Doerschuk, C.M., Hynes, R.O., and Baltimore, D. (2001). Targeted mutation of TNF receptor I rescues the RelA-deficient mouse and reveals a critical role for NF-kappa B in leukocyte recruitment. The Journal of Immunology *167*, 1592–1600.

Alon, R., and Feigelson, S. (2002). From rolling to arrest on blood vessels: leukocyte tap dancing on endothelial integrin ligands and chemokines at sub-second contacts. Seminars in Immunology *14*, 93–104.

Anders, S., and Huber, W. (2010). Differential expression analysis for sequence count data. Genome Biol. *11*, R106.

Anderson, M.S., Venanzi, E.S., Klein, L., Chen, Z., Berzins, S.P., Turley, S.J., Boehmer, von, H., Bronson, R., Dierich, A., Benoist, C., et al. (2002). Projection of an immunological self shadow within the thymus by the aire protein. Science *298*, 1395–1401.

Attar, R.M., Macdonald-Bravo, H., Raventos-Suarez, C., Durham, S.K., and Bravo, R. (1998). Expression of constitutively active IkappaB beta in T cells of transgenic mice: persistent NF-kappaB activity is required for T-cell immune responses. Mol. Cell. Biol. *18*, 477–487.

Bachmaier, K., Krawczyk, C., Kozieradzki, I., Kong, Y.-Y., Sasaki, T., Oliveira-dos-Santos, A., Mariathasan, S., Bouchard, D., Wakeham, A., Itie, A., et al. (2000). Negative regulation of lymphocyte activation and autoimmunity by the molecular adaptor Cbl-b. Nature *403*, 211–216.

Bankovich, A.J., Shiow, L.R., and Cyster, J.G. (2010). CD69 suppresses sphingosine 1-phosophate receptor-1 (S1P1) function through interaction with

membrane helix 4. J. Biol. Chem. *285*, 22328–22337.

Batista, F.D., and Harwood, N.E. (2009). The who, how and where of antigen presentation to B cells. Nat Rev Immunol *9*, 15–27.

Beg, A.A., and Baltimore, D. (1996). An essential role for NF-kappaB in preventing TNF-alpha-induced cell death. Science *274*, 782–784.

Beg, A.A., Sha, W.C., Bronson, R.T., Ghosh, S., and Baltimore, D. (1995). Embryonic lethality and liver degeneration in mice lacking the RelA component of NF-kappa B. Nature *376*, 167–170.

Benetatos, C.A., Mitsuuchi, Y., Burns, J.M., Neiman, E.M., Condon, S.M., Yu, G., Seipel, M.E., Kapoor, G.S., LaPorte, M.G., Rippin, S.R., et al. (2014). Birinapant (TL32711), a bivalent SMAC mimetic, targets TRAF2-associated cIAPs, abrogates TNF-induced NF-κB activation, and is active in patient-derived xenograft models. Mol. Cancer Ther. *13*, 867–879.

Benz, C., Heinzel, K., and Bleul, C.C. (2004). Homing of immature thymocytes to the subcapsular microenvironment within the thymus is not an absolute requirement for T cell development. Eur. J. Immunol. *34*, 3652–3663.

Berger, S.B., Kasparcova, V., Hoffman, S., Swift, B., Dare, L., Schaeffer, M., Capriotti, C., Cook, M., Finger, J., Hughes-Earle, A., et al. (2014). Cutting Edge: RIP1 kinase activity is dispensable for normal development but is a key regulator of inflammation in SHARPIN-deficient mice. J. Immunol. *192*, 5476–5480.

Bhatia, S.K., Tygrett, L.T., Grabstein, K.H., and Waldschmidt, T.J. (1995). The effect of in vivo IL-7 deprivation on T cell maturation. Journal of Experimental Medicine *181*, 1399–1409.

Black, R.A., Rauch, C.T., Kozlosky, C.J., Peschon, J.J., Slack, J.L., Wolfson, M.F., Castner, B.J., Stocking, K.L., Reddy, P., Srinivasan, S., et al. (1997). A metalloproteinase disintegrin that releases tumour-necrosis factor-alpha from cells. Nature *385*, 729–733.

Blattman, J.N., Antia, R., Sourdive, D.J.D., Wang, X., Kaech, S.M., Murali-Krishna, K., Altman, J.D., and Ahmed, R. (2002). Estimating the precursor frequency of naive antigen-specific CD8 T cells. Journal of Experimental Medicine *195*, 657–664.

Boothby, M.R., Mora, A.L., Scherer, D.C., Brockman, J.A., and Ballard, D.W. (1997). Perturbation of the T lymphocyte lineage in transgenic mice expressing a constitutive repressor of nuclear factor (NF)-kappaB. Journal of Experimental Medicine *185*, 1897–1907.

Borgulya, P., Kishi, H., Uematsu, Y., and Boehmer, von, H. (1992). Exclusion and inclusion of α and β T cell receptor alleles. Cell *69*, 529–537.

Borish, L.C., and Steinke, J.W. (2003). 2. Cytokines and chemokines. Journal of Allergy and Clinical Immunology *111*, S460–S475.

Borner, C. (2003). The Bcl-2 protein family: sensors and checkpoints for life-or-death decisions. Mol. Immunol. *39*, 615–647.

Borowski, C., Martin, C., Gounari, F., Haughn, L., Aifantis, I., Grassi, F., and Boehmer, H.V. (2002). On the brink of becoming a T cell. Current Opinion in Immunology *14*, 200–206.

Bradley, L.M., Haynes, L., and Swain, S.L. (2005). IL-7: maintaining T-cell memory and achieving homeostasis. Trends Immunol. *26*, 172–176.

Brenner, D., Blaser, H., and Mak, T.W. (2015). Regulation of tumour necrosis factor signalling: live or let die. Nat Rev Immunol *15*, 362–374.

Brockman, J.A., Scherer, D.C., McKinsey, T.A., Hall, S.M., Qi, X., Lee, W.Y., and Ballard, D.W. (1995). Coupling of a signal response domain in I kappa B alpha to multiple pathways for NF-kappa B activation. Mol. Cell. Biol. *15*, 2809–2818.

Bruin, J.E., Gerstein, H.C., Morrison, K.M., and Holloway, A.C. (2008). Increased pancreatic beta-cell apoptosis following fetal and neonatal exposure to nicotine is mediated via the mitochondria. Toxicol. Sci. *103*, 362–370.

Buentke, E. (2006). Do CD8 effector cells need IL-7R expression to become resting memory cells? Blood *108*, 1949–1956.

Bullard, D.C., Kunkel, E.J., Kubo, H., Hicks, M.J., Lorenzo, I., Doyle, N.A., Doerschuk, C.M., Ley, K., and Beaudet, A.L. (1996). Infectious susceptibility and severe deficiency of leukocyte rolling and recruitment in E-selectin and P-selectin double mutant mice. Journal of Experimental Medicine *183*, 2329–2336.

Burkly, L., Hession, C., Ogata, L., Reilly, C., Marconi, L.A., Olson, D., Tizard, R., Cate, R., and Lo, D. (1995). Expression of relB is required for the development of thymic medulla and dendritic cells. Nature *373*, 531–536.

Burnet, F.M. (1976). A Modification of Jerne's Theory of Antibody Production using the Concept of Clonal Selection. CA a Cancer Journal for Clinicians *26*, 119–121.

Caamano, J.H., Rizzo, C.A., Durham, S.K., Barton, D.S., Raventos-Suarez, C., Snapper, C.M., and Bravo, R. (1998). Nuclear factor (NF)-kappa B2 (p100/p52) is required for normal splenic microarchitecture and B cell-mediated immune responses. Journal of Experimental Medicine *187*, 185–196.

Cantrell, D.A. (2003). GTPases and T cell activation. Immunol. Rev. *192*, 122–130.

Cao, Y., Bonizzi, G., Seagroves, T.N., Greten, F.R., Johnson, R., Schmidt, E.V., and Karin, M. (2001). IKKalpha provides an essential link between RANK signaling and cyclin D1 expression during mammary gland development. Cell *107*, 763–775.

Carswell, E.A., Old, L.J., Kassel, R.L., Green, S., Fiore, N., and Williamson, B. (1975). An endotoxin-induced serum factor that causes necrosis of tumors. Proc. Natl. Acad. Sci. U.S.a. *72*, 3666–3670.

Cash, H.L., Whitham, C.V., Behrendt, C.L., and Hooper, L.V. (2006). Symbiotic bacteria direct expression of an intestinal bactericidal lectin. Science *313*, 1126–1130.

Caux, C., Ait-Yahia, S., Chemin, K., de Bouteiller, O., Dieu-Nosjean, M.C., Homey, B., Massacrier, C., Vanbervliet, B., Zlotnik, A., and Vicari, A. (2000). Dendritic cell biology and regulation of dendritic cell trafficking by chemokines. Springer Semin. Immunopathol. *22*, 345–369.

Ceredig, R., and Rolink, T. (2002). Opinion: A positive look at double-negative thymocytes. Nat Rev Immunol *2*, 888–897.

Chan, A.C., Dalton, M., Johnson, R., Kong, G.H., Wang, T., Thoma, R., and Kurosaki, T. (1995). Activation of ZAP-70 kinase activity by phosphorylation of tyrosine 493 is required for lymphocyte antigen receptor function. Embo J. *14*, 2499–2508.

Chang, D.W., Xing, Z., Capacio, V.L., Peter, M.E., and Yang, X. (2003). Interdimer processing mechanism of procaspase-8 activation. Embo J. *22*, 4132–4142.

Chatzidakis, I., and Mamalaki, C. (2010). T cells as sources and targets of TNF: implications for immunity and autoimmunity. Curr. Dir. Autoimmun. *11*, 105–118.

Chen, G., and Goeddel, D.V. (2002). TNF-R1 signaling: a beautiful pathway. Science *296*, 1634–1635.

Chen, X., Willette-Brown, J., Wu, X., Hu, Y., Howard, O.M.Z., Hu, Y., and Oppenheim, J.J. (2015). IKKα is required for the homeostasis of regulatory T cells and for the expansion of both regulatory and effector CD4 T cells. Faseb J. *29*, 443–454.

Chen, X., Wu, X., Zhou, Q., Howard, O.M.Z., Netea, M.G., and Oppenheim, J.J. (2013). TNFR2 is critical for the stabilization of the CD4+Foxp3+ regulatory T. cell phenotype in the inflammatory environment. J. Immunol. *190*, 1076–1084.

Chen, Z.J., Parent, L., and Maniatis, T. (1996). Site-specific phosphorylation of IkappaBalpha by a novel ubiquitination-dependent protein kinase activity. Cell *84*, 853–862.

Chien, Y.-H., Gascoigne, N.R.J., Kavaler, J., Lee, N.E., and Davis, M.M. (1984). Somatic recombination in a murine T-cell receptor gene. , Published Online: 24 May 1984; | Doi:10.1038/309322a0 *309*, 322–326.

Chinnaiyan, A.M., O'Rourke, K., Tewari, M., and Dixit, V.M. (1995). FADD, a novel death domain-containing protein, interacts with the death domain of Fas and initiates apoptosis. Cell *81*, 505–512.

Christofferson, D.E., Li, Y., and Yuan, J. (2014). Control of life-or-death decisions by RIP1 kinase. Annu. Rev. Physiol. *76*, 129–150.

Clark, K., Peggie, M., Plater, L., Sorcek, R.J., Young, E.R.R., Madwed, J.B., Hough, J., McIver, E.G., and Cohen, P. (2011). Novel cross-talk within the IKK family controls innate immunity. Biochem. J. *434*, 93–104.

Claudio, E., Brown, K., and Siebenlist, U. (2006). NF-|[kappa]|B guides the survival and differentiation of developing lymphocytes. *13*, 697–701.

Conrad, M., Angeli, J.P.F., Vandenabeele, P., and Stockwell, B.R. (2016). Regulated necrosis: disease relevance and therapeutic opportunities. Nat Rev Drug Discov.

Conte, D., Liston, P., Wong, J.W., Wright, K.E., and Korneluk, R.G. (2001). Thymocyte-targeted overexpression of xiap transgene disrupts T lymphoid apoptosis and maturation. Pnas *98*, 5049–5054.

Coquet, J.M., Ribot, J.C., Bąbała, N., Middendorp, S., van der Horst, G., Xiao, Y., Neves, J.F., Fonseca-Pereira, D., Jacobs, H., Pennington, D.J., et al. (2013). Epithelial and dendritic cells in the thymic medulla promote CD4+Foxp3+ regulatory T cell development via the CD27-CD70 pathway. J. Exp. Med. *210*, 715–728.

Cose, S. (2007). T- cell migration: a naive paradigm? Immunology *120*, 1–7.

Courtois, G., and Gilmore, T.D. (2006). Mutations in the NF-kappaB signaling pathway: implications for human disease. Oncogene *25*, 6831–6843.

Croft, M., Benedict, C.A., and Ware, C.F. (2013). Clinical targeting of the TNF and TNFR superfamilies. Nat Rev Drug Discov *12*, 147–168.

Davis, D.M. (2013). The Compatibility Gene (Penguin UK).

de Boer, J., Williams, A., Skavdis, G., Harker, N., Coles, M., Tolaini, M., Norton, T., Williams, K., Roderick, K., Potocnik, A.J., et al. (2003). Transgenic mice with hematopoietic and lymphoid specific expression of Cre. Eur. J. Immunol. *33*, 314–325.

De Smet, K., and Contreras, R. (2005). Human Antimicrobial Peptides: Defensins, Cathelicidins and Histatins. Biotechnol Lett *27*, 1337–1347.

Degterev, A., Hitomi, J., Germscheid, M., Ch'en, I.L., Korkina, O., Teng, X., Abbott, D., Cuny, G.D., Yuan, C., Wagner, G., et al. (2008). Identification of RIP1 kinase as a specific cellular target of necrostatins. Nat. Chem. Biol. *4*, 313–321.

Delhase, M., Hayakawa, M., Chen, Y., and Karin, M. (1999). Positive and negative regulation of IkappaB kinase activity through IKKbeta subunit phosphorylation. Science *284*, 309–313.

Dellinger, R.P. (2003). Inflammation and coagulation: implications for the septic

patient. Clin. Infect. Dis. *36*, 1259–1265.

Deng, L., Wang, C., Spencer, E., Yang, L., Braun, A., You, J., Slaughter, C., Pickart, C., and Chen, Z.J. (2000). Activation of the IkappaB kinase complex by TRAF6 requires a dimeric ubiquitin-conjugating enzyme complex and a unique polyubiquitin chain. Cell *103*, 351–361.

Devin, A., Lin, Y., Yamaoka, S., Li, Z., Karin, M., and Liu Zg (2001). The alpha and beta subunits of IkappaB kinase (IKK) mediate TRAF2-dependent IKK recruitment to tumor necrosis factor (TNF) receptor 1 in response to TNF. Mol. Cell. Biol. *21*, 3986–3994.

DiDonato, J.A., Hayakawa, M., Rothwarf, D.M., Zandi, E., and Karin, M. (1997). A cytokine-responsive IkappaB kinase that activates the transcription factor NF-kappaB. Nature *388*, 548–554.

Doi, T.S., Marino, M.W., Takahashi, T., Yoshida, T., Sakakura, T., Old, L.J., and Obata, Y. (1999). Absence of tumor necrosis factor rescues RelA-deficient mice from embryonic lethality. Proc. Natl. Acad. Sci. U.S.a. *96*, 2994–2999.

Dondelinger, Y., Aguileta, M.A., Goossens, V., Dubuisson, C., Grootjans, S., Dejardin, E., Vandenabeele, P., and Bertrand, M.J.M. (2013). RIPK3 contributes to TNFR1-mediated RIPK1 kinase-dependent apoptosis in conditions of cIAP1/2 depletion or TAK1 kinase inhibition. *20*, 1381–1392.

Dong, C., and Martinez, G.J. (2010). T cells: the usual subsets. Nat Rev Immunol.

Dzhagalov, I., and Phee, H. (2012). How to find your way through the thymus: a practical guide for aspiring T cells. Cell. Mol. Life Sci. *69*, 663–682.

Ea, C.-K., Deng, L., Xia, Z.-P., Pineda, G., and Chen, Z.J. (2006). Activation of IKK by TNFalpha requires site-specific ubiquitination of RIP1 and polyubiquitin binding by NEMO. Mol. Cell *22*, 245–257.

Egawa, T., Albrecht, B., Favier, B., Sunshine, M.-J., Mirchandani, K., O'Brien, W., Thome, M., and Littman, D.R. (2003). Requirement for CARMA1 in antigen receptor-induced NF-kappa B activation and lymphocyte proliferation. Curr. Biol. *13*, 1252–1258.

Egawa, T., Kreslavsky, T., Littman, D.R., and Boehmer, von, H. (2008). Lineage Diversion of T Cell Receptor Transgenic Thymocytes Revealed by Lineage Fate Mapping. PLoS ONE *3*, e1512.

Egawa, T., Tillman, R.E., Naoe, Y., Taniuchi, I., and Littman, D.R. (2007). The role of the Runx transcription factors in thymocyte differentiation and in homeostasis of naive T cells. Journal of Experimental Medicine *204*, 1945–1957.

Ehlers, S., Kutsch, S., Ehlers, E.M., Benini, J., and Pfeffer, K. (2000). Lethal granuloma disintegration in mycobacteria-infected TNFRp55-/- mice is dependent on T cells and IL-12. The Journal of Immunology *165*, 483–492.

Elmore, S. (2007). Apoptosis: a review of programmed cell death. Toxicol Pathol *35*, 495–516.

Enari, M., Sakahira, H., Yokoyama, H., Okawa, K., Iwamatsu, A., and Nagata, S. (1998). A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. Nature *391*, 43–50.

Esslinger, C.W., Jongeneel, C.V., and MacDonald, H.R. (1998). Survival-independent function of NF-kappaB/Rel during late stages of thymocyte differentiation. Mol. Immunol. *35*, 847–852.

Esslinger, C.W., Wilson, A., Sordat, B., Beermann, F., and Jongeneel, C.V. (1997). Abnormal T lymphocyte development induced by targeted overexpression of IkappaB alpha. The Journal of Immunology *158*, 5075–5078.

Estornes, Y., and Bertrand, M.J.M. (2015). IAPs, regulators of innate immunity and inflammation. Semin. Cell Dev. Biol. *39*, 106–114.

Farber, D.L., Yudanin, N.A., and Restifo, N.P. (2013). Human memory T cells: generation, compartmentalization and homeostasis. Nat Rev Immunol *14*, 24–35.

Fearon, D.T., and Locksley, R.M. (1996). The instructive role of innate immunity in the acquired immune response. Science *272*, 50–53.

Feng, S., Yang, Y., Mei, Y., Ma, L., Zhu, D.-E., Hoti, N., Castanares, M., and Wu, M. (2007). Cleavage of RIP3 inactivates its caspase-independent apoptosis pathway by removal of kinase domain. Cell. Signal. *19*, 2056–2067.

Festjens, N., Vanden Berghe, T., Cornelis, S., and Vandenabeele, P. (2007). RIP1, a kinase on the crossroads of a cell's decision to live or die. *14*, 400–410.

Förster, R., Braun, A., and Worbs, T. (2012). Lymph node homing of T cells and dendritic cells via afferent lymphatics. Trends Immunol. *33*, 271–280.

Franzoso, G., Carlson, L., Poljak, L., Shores, E.W., Epstein, S., Leonardi, A., Grinberg, A., Tran, T., Scharton-Kersten, T., Anver, M., et al. (1998). Mice deficient in nuclear factor (NF)-kappa B/p52 present with defects in humoral responses, germinal center reactions, and splenic microarchitecture. Journal of Experimental Medicine *187*, 147–159.

Freeden-Jeffry, von, U., Vieira, P., Lucian, L.A., McNeil, T., Burdach, S.E., and Murray, R. (1995). Lymphopenia in interleukin (IL)-7 gene-deleted mice identifies IL-7 as a nonredundant cytokine. Journal of Experimental Medicine *181*, 1519–1526.

Freitas, A.A., and Rocha, B. (2003). Population Biology of Lymphocytes: The Flight for Survival. Http://Dx.Doi.org/10.1146/Annurev.Immunol.18.1.83 *18*, 83–111.

Fujita, H., Rahighi, S., Akita, M., Kato, R., Sasaki, Y., Wakatsuki, S., and Iwai, K. (2014). Mechanism underlying IκB kinase activation mediated by the linear

ubiquitin chain assembly complex. Mol. Cell. Biol. *34*, 1322–1335.

Gaffen, S.L. (2001). Signaling domains of the interleukin 2 receptor. Cytokine *14*, 63–77.

Gareus, R., Huth, M., Breiden, B., Nenci, A., Rösch, N., Haase, I., Bloch, W., Sandhoff, K., and Pasparakis, M. (2007). Normal epidermal differentiation but impaired skin-barrier formation upon keratinocyte-restricted IKK1 ablation. Nat. Cell Biol. *9*, 461–469.

Geiselhart, L.A., Humphries, C.A., Gregorio, T.A., Mou, S., Subleski, J., and Komschlies, K.L. (2001). IL-7 administration alters the CD4:CD8 ratio, increases T cell numbers, and increases T cell function in the absence of activation. The Journal of Immunology *166*, 3019–3027.

Gerlach, B., Cordier, S.M., Schmukle, A.C., Emmerich, C.H., Rieser, E., Haas, T.L., Webb, A.I., Rickard, J.A., Anderton, H., Wong, W.W.-L., et al. (2011). Linear ubiquitination prevents inflammation and regulates immune signalling. Nature *471*, 591–596.

Germain, R.N. (2002). T-cell development and the CD4-CD8 lineage decision. Nat Rev Immunol *2*, 309–322.

Gerondakis, S., Fulford, T.S., Messina, N.L., and Grumont, R.J. (2013). NF-κB control of T cell development. Nat Immunol *15*, 15–25.

Gershon, R.K., and Kondo, K. (1970). Cell interactions in the induction of tolerance: the role of thymic lymphocytes. Immunology *18*, 723–737.

Ghosh, S., May, M.J., and Kopp, E.B. (1998). NF-kappa B and Rel proteins: evolutionarily conserved mediators of immune responses. Annu. Rev. Immunol. *16*, 225–260.

Ghosh, S., and Karin, M. (2002). Missing pieces in the NF-kappaB puzzle. Cell *109 Suppl*, S81–S96.

Gilmore, T.D., and Herscovitch, M. (2006). Inhibitors of NF-kappaB signaling: 785 and counting. Oncogene *25*, 6887–6899.

Girardi, M. (2006). Immunosurveillance and Immunoregulation by |[gamma]||[delta]| T Cells. Journal of Investigative Dermatology *126*, 25–31.

Giroir, B.P., Brown, T., and Beutler, B. (1992). Constitutive synthesis of tumor necrosis factor in the thymus. Pnas *89*, 4864–4868.

Glick, B., Chang, T.S., and Jaap, R.G. (1956). The Bursa of Fabricius and Antibody Production. Poultry Science *35*, 224–225.

Godfrey, D.I., Kennedy, J., Suda, T., and Zlotnik, A. (1993). A developmental pathway involving four phenotypically and functionally distinct subsets of CD3-CD4-CD8- triple-negative adult mouse thymocytes defined by CD44 and CD25 expression. The Journal of Immunology *150*, 4244–4252.

Grabstein, K.H., Waldschmidt, T.J., Finkelman, F.D., Hess, B.W., Alpert, A.R., Boiani, N.E., Namen, A.E., and Morrissey, P.J. (1993). Inhibition of murine B and T lymphopoiesis in vivo by an anti-interleukin 7 monoclonal antibody. Journal of Experimental Medicine *178*, 257–264.

Grakoui, A., Bromley, S.K., Sumen, C., Davis, M.M., Shaw, A.S., Allen, P.M., and Dustin, M.L. (1999). The immunological synapse: a molecular machine controlling T cell activation. Science *285*, 221–227.

Granger, D.N., and Senchenkova, E. (2010). Inflammation and the Microcirculation (San Rafael (CA): Morgan & Claypool Life Sciences).

Greenwald, R.J., Freeman, G.J., and Sharpe, A.H. (2004). THE B7 FAMILY REVISITED. Http://Dx.Doi.org/10.1146/Annurev.Immunol.23.021704.115611 *23*, 515–548.

Gros, P., Milder, F.J., and Janssen, B.J.C. (2008). Complement driven by conformational changes. Nat Rev Immunol *8*, 48–58.

Grossmann, M., Metcalf, D., Merryfull, J., Beg, A., Baltimore, D., and Gerondakis, S. (1999). The combined absence of the transcription factors Rel and RelA leads to multiple hemopoietic cell defects. Proc. Natl. Acad. Sci. U.S.a. *96*, 11848–11853.

Gu, H., Marth, J.D., Orban, P.C., Mossmann, H., and Rajewsky, K. (1994). Deletion of a DNA polymerase beta gene segment in T cells using cell type-specific gene targeting. Science *265*, 103–106.

Gudmundsdottir, H., Wells, A.D., and Turka, L.A. (1999). Dynamics and requirements of T cell clonal expansion in vivo at the single-cell level: effector function is linked to proliferative capacity. The Journal of Immunology *162*, 5212–5223.

Gugasyan, R., Horat, E., Kinkel, S.A., Ross, F., Grigoriadis, G., Gray, D., O'Keeffe, M., Berzins, S.P., Belz, G.T., Grumont, R.J., et al. (2012). The NF-κB1 transcription factor prevents the intrathymic development of CD8 T cells with memory properties. Embo J. *31*, 692–706.

Haas, T.L., Emmerich, C.H., Gerlach, B., Schmukle, A.C., Cordier, S.M., Rieser, E., Feltham, R., Vince, J., Warnken, U., Wenger, T., et al. (2009). Recruitment of the linear ubiquitin chain assembly complex stabilizes the TNF-R1 signaling complex and is required for TNF-mediated gene induction. Mol. Cell *36*, 831–844.

Han, J., Zhong, C.-Q., and Zhang, D.-W. (2011). Programmed necrosis: backup to and competitor with apoptosis in the immune system. Nat Immunol *12*, 1143–1149.

Hand, T.W., Cui, W., Jung, Y.W., Sefik, E., Joshi, N.S., Chandele, A., Liu, Y., and Kaech, S.M. (2010). Differential effects of STAT5 and PI3K/AKT signaling on effector and memory CD8 T-cell survival. Proc. Natl. Acad. Sci. U.S.a. *107*, 16601–16606.

Hasegawa, M., Fujimoto, Y., Lucas, P.C., Nakano, H., Fukase, K., Nuñez, G., and Inohara, N. (2008). A critical role of RICK/RIP2 polyubiquitination in Nod-induced NF-kappaB activation. Embo J. *27*, 373–383.

Hayden, M.S., and Ghosh, S. (2004). Signaling to NF-kappaB. Genes & Development *18*, 2195–2224.

Hayden, M.S., and Ghosh, S. (2008). Shared principles in NF-kappaB signaling. Cell *132*, 344–362.

Hayden, M.S., and Ghosh, S. (2012). NF-κB, the first quarter-century: remarkable progress and outstanding questions. Genes & Development *26*, 203–234.

Hayden, M.S., and Ghosh, S. (2014). Regulation of NF-κB by TNF family cytokines. Seminars in Immunology *26*, 253–266.

Hayden, M.S., West, A.P., and Ghosh, S. (2006). SnapShot: NF-kappaB signaling pathways. Cell *127*, 1286–1287.

Hayes, S.M., Li, L., and Love, P.E. (2005). TCR signal strength influences alphabeta/gammadelta lineage fate. Immunity *22*, 583–593.

He, X., He, X., Dave, V.P., Zhang, Y., Hua, X., Nicolas, E., Xu, W., Roe, B.A., and Kappes, D.J. (2005). The zinc finger transcription factor Th-POK regulates CD4 versus CD8 T-cell lineage commitment. Nature *433*, 826–833.

Hedrick, S.M. (2004). The acquired immune system: a vantage from beneath. Immunity *21*, 607–615.

Hendriks, J., Gravestein, L.A., Tesselaar, K., van Lier, R.A.W., Schumacher, T.N.M., and Borst, J. (2000). CD27 is required for generation and long-term maintenance of T cell immunity. Nat Immunol *1*, 433–440.

Herzenberg, L.A., Parks, D., Sahaf, B., Perez, O., Roederer, M., and Herzenberg, L.A. (2002). The history and future of the fluorescence activated cell sorter and flow cytometry: a view from Stanford. Clin. Chem. *48*, 1819–1827.

Hettmann, T., and Leiden, J.M. (2000). NF-kappa B is required for the positive selection of CD8+ thymocytes. The Journal of Immunology *165*, 5004–5010.

Heyman, B., Pilström, L., and Shulman, M.J. (1988). Complement activation is required for IgM-mediated enhancement of the antibody response. Journal of Experimental Medicine *167*, 1999–2004.

Hiscott, J., Nguyen, T.-L.A., Arguello, M., Nakhaei, P., and Paz, S. (2006). Manipulation of the nuclear factor-kappaB pathway and the innate immune response by viruses. Oncogene *25*, 6844–6867.

Hogan, P.G., Chen, L., Nardone, J., and Rao, A. (2003). Transcriptional regulation by calcium, calcineurin, and NFAT. Genes & Development *17*, 2205–

2232.

Hogquist, K.A. (2001). Signal strength in thymic selection and lineage commitment. Current Opinion in Immunology *13*, 225–231.

Honda, K., and Taniguchi, T. (2006). IRFs: master regulators of signalling by Toll-like receptors and cytosolic pattern-recognition receptors. Nat Rev Immunol *6*, 644–658.

Horwitz, B.H., Scott, M.L., Cherry, S.R., Bronson, R.T., and Baltimore, D. (1997). Failure of Lymphopoiesis after Adoptive Transfer of NF-κB–Deficient Fetal Liver Cells. Immunity *6*, 765–772.

Hozumi, N., and Tonegawa, S. (1976). Evidence for somatic rearrangement of immunoglobulin genes coding for variable and constant regions. Proc. Natl. Acad. Sci. U.S.a. *73*, 3628–3632.

Hsu, H., Huang, J., Shu, H.B., Baichwal, V., and Goeddel, D.V. (1996a). TNF-dependent recruitment of the protein kinase RIP to the TNF receptor-1 signaling complex. Immunity *4*, 387–396.

Hsu, H., Shu, H.B., Pan, M.G., and Goeddel, D.V. (1996b). TRADD-TRAF2 and TRADD-FADD interactions define two distinct TNF receptor 1 signal transduction pathways. Cell *84*, 299–308.

Hsu, H., Xiong, J., and Goeddel, D.V. (1995). The TNF receptor 1-associated protein TRADD signals cell death and NF-kappa B activation. Cell *81*, 495–504.

Hu, Y., Baud, V., Delhase, M., Zhang, P., Deerinck, T., Ellisman, M., Johnson, R., and Karin, M. (1999). Abnormal morphogenesis but intact IKK activation in mice lacking the IKKalpha subunit of IkappaB kinase. Science *284*, 316–320.

Hu, Y., Baud, V., Oga, T., Kim, K.I., Yoshida, K., and Karin, M. (2001). IKKalpha controls formation of the epidermis independently of NF-kappaB. Nature *410*, 710–714.

Huesmann, M., Scott, B., Kisielow, P., and Boehmer, von, H. (1991). Kinetics and efficacy of positive selection in the thymus of normal and T cell receptor transgenic mice. Cell *66*, 533–540.

Huse, M. (2009). The T-cell-receptor signaling network. J. Cell. Sci. *122*, 1269–1273.

Huxford, T., and Ghosh, G. (2009). A structural guide to proteins of the NF-kappaB signaling module. Cold Spring Harbor Perspectives in Biology *1*, a000075–a000075.

Huynh, Q.K., Boddupalli, H., Rouw, S.A., Koboldt, C.M., Hall, T., Sommers, C., Hauser, S.D., Pierce, J.L., Combs, R.G., Reitz, B.A., et al. (2000). Characterization of the recombinant IKK1/IKK2 heterodimer. Mechanisms regulating kinase activity. Journal of Biological Chemistry *275*, 25883–25891.

Isomura, I., Palmer, S., Grumont, R.J., Bunting, K., Hoyne, G., Wilkinson, N., Banerjee, A., Proietto, A., Gugasyan, R., Wu, L., et al. (2009). c-Rel is required for the development of thymic Foxp3+ CD4 regulatory T cells. J. Exp. Med. *206*, 3001–3014.

Iwashima, M., Irving, B.A., Van Oers, N.S., Chan, A.C., and Weiss, A. (1994). Sequential interactions of the TCR with two distinct cytoplasmic tyrosine kinases. Science *263*, 1136–1139.

Jameson, S.C. (2005). T cell homeostasis: keeping useful T cells alive and live T cells useful. Seminars in Immunology *17*, 231–237.

Jerne, N.K. (1955). THE NATURAL-SELECTION THEORY OF ANTIBODY FORMATION. Proc. Natl. Acad. Sci. U.S.a. *41*, 849–857.

Jiang, Y., Woronicz, J.D., Liu, W., and Goeddel, D.V. (1999). Prevention of constitutive TNF receptor 1 signaling by silencer of death domains. Science *283*, 543–546.

Jimi, E., Strickland, I., Voll, R.E., Long, M., and Ghosh, S. (2008). Differential role of the transcription factor NF-kappaB in selection and survival of CD4+ and CD8+ thymocytes. Immunity *29*, 523–537.

Johnston, B., and Butcher, E.C. (2002). Chemokines in rapid leukocyte adhesion triggering and migration. Seminars in Immunology *14*, 83–92.

Jordan, M.S., Boesteanu, A., Reed, A.J., Petrone, A.L., Holenbeck, A.E., Lerman, M.A., Naji, A., and Caton, A.J. (2001). Thymic selection of CD4+CD25+ regulatory T cells induced by an agonist self-peptide. Nat Immunol *2*, 301–306.

Jordan, M.S., Singer, A.L., and Koretzky, G.A. (2003). Adaptors as central mediators of signal transduction in immune cells. Nat Immunol *4*, 110–116.

Kabelitz, D. (2007). Expression and function of Toll-like receptors in T lymphocytes. Current Opinion in Immunology *19*, 39–45.

Kaisho, T., Takeda, K., Tsujimura, T., Kawai, T., Nomura, F., Terada, N., and Akira, S. (2001). IkappaB kinase alpha is essential for mature B cell development and function. Journal of Experimental Medicine *193*, 417–426.

Karin, M., and Lin, A. (2002). NF-kappaB at the crossroads of life and death. Nat Immunol *3*, 221–227.

Kelliher, M.A., Grimm, S., Ishida, Y., Kuo, F., Stanger, B.Z., and Leder, P. (1998). The death domain kinase RIP mediates the TNF-induced NF-kappaB signal. Immunity *8*, 297–303.

Kerdiles, Y.M., Beisner, D.R., Tinoco, R., Dejean, A.S., Castrillon, D.H., DePinho, R.A., and Hedrick, S.M. (2009). Foxo1 links homing and survival of naive T cells by regulating L-selectin, CCR7 and interleukin 7 receptor. Nat Immunol *10*, 176–184.

Kincade, P.W., and Kelsoe, G. (2003). A birthday for B cells: Lymphopoiesis II, a scientific symposium honoring Max Cooper. Nat Immunol *4*, 1155–1157.

Kirberg, J., Berns, A., and Boehmer, von, H. (1997). Peripheral T cell survival requires continual ligation of the T cell receptor to major histocompatibility complex-encoded molecules. Journal of Experimental Medicine *186*, 1269–1275.

Kirisako, T., Kamei, K., Murata, S., Kato, M., Fukumoto, H., Kanie, M., Sano, S., Tokunaga, F., Tanaka, K., and Iwai, K. (2006). A ubiquitin ligase complex assembles linear polyubiquitin chains. Embo J. *25*, 4877–4887.

Kishimoto, A., Takai, Y., Mori, T., Kikkawa, U., and Nishizuka, Y. (1980). Activation of calcium and phospholipid-dependent protein kinase by diacylglycerol, its possible relation to phosphatidylinositol turnover. Journal of Biological Chemistry *255*, 2273–2276.

Klein, L., Kyewski, B., Allen, P.M., and Hogquist, K.A. (2014). Positive and negative selection of the T cell repertoire: what thymocytes see (and don't see). Nat Rev Immunol *14*, 377–391.

Kolaczkowska, E., and Kubes, P. (2013). Neutrophil recruitment and function in health and inflammation. Nat Rev Immunol *13*, 159–175.

Kontgen, F., Grumont, R.J., Strasser, A., Metcalf, D., Li, R., Tarlinton, D., and Gerondakis, S. (1995). Mice lacking the c-rel proto-oncogene exhibit defects in lymphocyte proliferation, humoral immunity, and interleukin-2 expression. Genes & Development *9*, 1965–1977.

Kopf, M., Ruedl, C., Schmitz, N., Gallimore, A., Lefrang, K., Ecabert, B., Odermatt, B., and Bachmann, M.F. (1999). OX40-Deficient Mice Are Defective in Th Cell Proliferation but Are Competent in Generating B Cell and CTL Responses after Virus Infection. Immunity *11*, 699–708.

Kreslavsky, T., Gleimer, M., Garbe, A.I., and Boehmer, von, H. (2010). αβ versus γδ fate choice: counting the T-cell lineages at the branch point. Immunol. Rev. *238*, 169–181.

Kreuz, S., Siegmund, D., Rumpf, J.-J., Samel, D., Leverkus, M., Janssen, O., Häcker, G., Dittrich-Breiholz, O., Kracht, M., Scheurich, P., et al. (2004). NFkappaB activation by Fas is mediated through FADD, caspase-8, and RIP and is inhibited by FLIP. J Cell Biol *166*, 369–380.

Kwak, J.-H., Jung, J.-K., and Lee, H. (2011). Nuclear factor-kappa B inhibitors; a patent review (2006-2010). Expert Opin Ther Pat *21*, 1897–1910.

Labrecque, N., Whitfield, L.S., Obst, R., Waltzinger, C., Benoist, C., and Mathis, D. (2001). How Much TCR Does a T Cell Need? Immunity *15*, 71–82.

Lattin, J., Zidar, D.A., Schroder, K., Kellie, S., Hume, D.A., and Sweet, M.J. (2007). G-protein-coupled receptor expression, function, and signaling in macrophages. J Leukoc Biol *82*, 16–32.

Lauritsen, J.P.H., Wong, G.W., Lee, S.-Y., Lefebvre, J.M., Ciofani, M., Rhodes, M., Kappes, D.J., Zúñiga-Pflücker, J.C., and Wiest, D.L. (2009). Marked induction of the helix-loop-helix protein Id3 promotes the gammadelta T cell fate and renders their functional maturation Notch independent. Immunity *31*, 565–575.

Lawrence, T. (2009). The nuclear factor NF-kappaB pathway in inflammation. Cold Spring Harbor Perspectives in Biology *1*, a001651–a001651.

Lee, F.S., Hagler, J., Chen, Z.J., and Maniatis, T. (1997). Activation of the IkappaB alpha kinase complex by MEKK1, a kinase of the JNK pathway. Cell *88*, 213–222.

Lee, P.P., Fitzpatrick, D.R., Beard, C., Jessup, H.K., Lehar, S., Makar, K.W., Pérez-Melgosa, M., Sweetser, M.T., Schlissel, M.S., Nguyen, S., et al. (2001). A critical role for Dnmt1 and DNA methylation in T cell development, function, and survival. Immunity *15*, 763–774.

Lee, T.H., Shank, J., Cusson, N., and Kelliher, M.A. (2004). The kinase activity of Rip1 is not required for tumor necrosis factor-alpha-induced IkappaB kinase or p38 MAP kinase activation or for the ubiquitination of Rip1 by Traf2. Journal of Biological Chemistry *279*, 33185–33191.

Legarda-Addison, D., Hase, H., O'Donnell, M.A., and Ting, A.T. (2009). NEMO/IKKgamma regulates an early NF-kappaB-independent cell-death checkpoint during TNF signaling. *16*, 1279–1288.

Lemmon, M.A. (2003). Phosphoinositide Recognition Domains. Traffic *4*, 201–213.

Leonard, W.J., and O'Shea, J.J. (1998). Jaks and STATs: biological implications. Annu. Rev. Immunol. *16*, 293–322.

Lesley, R., Kelly, L.M., Xu, Y., and Cyster, J.G. (2006). Naive CD4 T cells constitutively express CD40L and augment autoreactive B cell survival. Pnas *103*, 10717–10722.

Li, Q., Estepa, G., Memet, S., Israel, A., and Verma, I.M. (2000). Complete lack of NF-kappaB activity in IKK1 and IKK2 double-deficient mice: additional defect in neurulation. Genes & Development *14*, 1729–1733.

Li, Q., Lu, Q., Hwang, J.Y., Büscher, D., Lee, K.F., Izpisua-Belmonte, J.C., and Verma, I.M. (1999a). IKK1-deficient mice exhibit abnormal development of skin and skeleton. Genes & Development *13*, 1322–1328.

Li, Q., Van Antwerp, D., Mercurio, F., Lee, K.F., and Verma, I.M. (1999b). Severe liver degeneration in mice lacking the IkappaB kinase 2 gene. Science *284*, 321–325.

Li, Q.-J., Dinner, A.R., Qi, S., Irvine, D.J., Huppa, J.B., Davis, M.M., and Chakraborty, A.K. (2004). CD4 enhances T cell sensitivity to antigen by coordinating Lck accumulation at the immunological synapse. Nat Immunol *5*,

791–799.

Li, Z.W., Chu, W., Hu, Y., Delhase, M., Deerinck, T., Ellisman, M., Johnson, R., and Karin, M. (1999c). The IKKbeta subunit of IkappaB kinase (IKK) is essential for nuclear factor kappaB activation and prevention of apoptosis. Journal of Experimental Medicine *189*, 1839–1845.

Li, Z.-W., Omori, S.A., Labuda, T., Karin, M., and Rickert, R.C. (2003). IKK beta is required for peripheral B cell survival and proliferation. The Journal of Immunology *170*, 4630–4637.

Lim, J.P., and Gleeson, P.A. (2011). Macropinocytosis: an endocytic pathway for internalising large gulps. Immunol Cell Biol *89*, 836–843.

Lin, A.E., and Mak, T.W. (2007). The role of E3 ligases in autoimmunity and the regulation of autoreactive T cells. Current Opinion in Immunology *19*, 665–673.

Lin, J., and Weiss, A. (2001). T cell receptor signalling. J. Cell. Sci. *114*, 243–244.

Lin, Y., Devin, A., Rodriguez, Y., and Liu, Z.G. (1999). Cleavage of the death domain kinase RIP by caspase-8 prompts TNF-induced apoptosis. Genes & Development *13*, 2514–2526.

Ling, L., Cao, Z., and Goeddel, D.V. (1998). NF-kappaB-inducing kinase activates IKK-alpha by phosphorylation of Ser-176. Proc. Natl. Acad. Sci. U.S.a. *95*, 3792–3797.

Littman, D.R., and Rudensky, A.Y. (2010). Th17 and Regulatory T Cells in Mediating and Restraining Inflammation. Cell *140*, 845–858.

Liu, H.-H., Xie, M., Schneider, M.D., and Chen, Z.J. (2006). Essential role of TAK1 in thymocyte development and activation. Proc. Natl. Acad. Sci. U.S.a. *103*, 11677–11682.

Liu, Y.-C., Penninger, J., and Karin, M. (2005). Immunity by ubiquitylation: a reversible process of modification. Nat Rev Immunol *5*, 941–952.

Lundberg, K., Heath, W., Kontgen, F., Carbone, F.R., and Shortman, K. (1995). Intermediate steps in positive selection: differentiation of CD4+8int TCRint thymocytes into CD4-8+TCRhi thymocytes. Journal of Experimental Medicine *181*, 1643–1651.

Ma, C.S., Deenick, E.K., Batten, M., and Tangye, S.G. (2012). The origins, function, and regulation of T follicular helper cells. J. Exp. Med. *209*, 1241–1253.

Mackay, F., and Schneider, P. (2009). Cracking the BAFF code. Nat Rev Immunol *9*, 491–502.

Maggi, E., Cosmi, L., Liotta, F., Romagnani, P., Romagnani, S., and Annunziato, F. (2005). Thymic regulatory T cells. Autoimmunity Reviews *4*,

579–586.

Mahmud, S.A., Manlove, L.S., Schmitz, H.M., Xing, Y., Wang, Y., Owen, D.L., Schenkel, J.M., Boomer, J.S., Green, J.M., Yagita, H., et al. (2014). Costimulation via the tumor-necrosis factor receptor superfamily couples TCR signal strength to the thymic differentiation of regulatory T cells. Nat Immunol *15*, 473–481.

Mahoney, D.J., Cheung, H.H., Mrad, R.L., Plenchette, S., Simard, C., Enwere, E., Arora, V., Mak, T.W., Lacasse, E.C., Waring, J., et al. (2008). Both cIAP1 and cIAP2 regulate TNFalpha-mediated NF-kappaB activation. Proc. Natl. Acad. Sci. U.S.a. *105*, 11778–11783.

Malissen, M., Trucy, J., Letourneur, F., Rebai, N., Dunn, D.E., Fitch, F.W., Hood, L., and Malissen, B. (1988). A T cell clone expresses two T cell receptor α genes but uses one αβ heterodimer for allorecognition and self MHC-restricted antigen recognition. Cell *55*, 49–59.

Mamalaki, C., Elliott, J., Norton, T., Yannoutsos, N., Townsend, A.R., Chandler, P., Simpson, E., and Kioussis, D. (1993). Positive and negative selection in transgenic mice expressing a T-cell receptor specific for influenza nucleoprotein and endogenous superantigen. Dev. Immunol. *3*, 159–174.

Markiewski, M.M., and Lambris, J.D. (2007). The Role of Complement in Inflammatory Diseases From Behind the Scenes into the Spotlight. The American Journal of Pathology *171*, 715–727.

Marschner, S., Freiberg, B.A., Kupfer, A., Hünig, T., and Finkel, T.H. (1999). Ligation of the CD4 receptor induces activation-independent down-regulation of L-selectin. Pnas *96*, 9763–9768.

McAllister-Lucas, L.M., Inohara, N., Lucas, P.C., Ruland, J., Benito, A., Li, Q., Chen, S., Chen, F.F., Yamaoka, S., Verma, I.M., et al. (2001). Bimp1, a MAGUK family member linking protein kinase C activation to Bcl10-mediated NF-kappaB induction. Journal of Biological Chemistry *276*, 30589–30597.

McCaughtry, T.M., Baldwin, T.A., Wilken, M.S., and Hogquist, K.A. (2008). Clonal deletion of thymocytes can occur in the cortex with no involvement of the medulla. J. Exp. Med. *205*, 2575–2584.

McCaughtry, T.M., Etzensperger, R., Alag, A., Tai, X., Kurtulus, S., Park, J.-H., Grinberg, A., Love, P., Feigenbaum, L., Erman, B., et al. (2012). Conditional deletion of cytokine receptor chains reveals that IL-7 and IL-15 specify CD8 cytotoxic lineage fate in the thymus. J. Exp. Med. *209*, 2263–2276.

Medawar, P.B. (1979). Advice To A Young Scientist (Basic Books).

Mercurio, F., and Manning, A.M. (1999). Multiple signals converging on NF-kappaB. Curr. Opin. Cell Biol. *11*, 226–232.

Michie, A.M., Trop, S., Wiest, D.L., and Zúñiga-Pflücker, J.C. (1999). Extracellular signal-regulated kinase (ERK) activation by the pre-T cell receptor

in developing thymocytes in vivo. Journal of Experimental Medicine *190*, 1647–1656.

Miller, J.F. (1961). Immunological function of the thymus. Lancet *2*, 748–749.

Miller, J.F.A.P. (2014). Revisiting thymus function. Front Immunol *5*, 411.

Miller, M.L., Mashayekhi, M., Chen, L., Zhou, P., Liu, X., Michelotti, M., Tramontini Gunn, N., Powers, S., Zhu, X., Evaristo, C., et al. (2014). Basal NF-κB controls IL-7 responsiveness of quiescent naïve T cells. Proc. Natl. Acad. Sci. U.S.a. *111*, 7397–7402.

Minakhina, S., and Steward, R. (2006). Nuclear factor-kappa B pathways in Drosophila. Oncogene *25*, 6749–6757.

Molina, T.J., Kishihara, K., Siderovskid, D.P., Van Ewijk, W., Narendran, A., Timms, E., Wakeham, A., Paige, C.J., Hartmann, K.U., Veillette, A., et al. (1992). Profound block in thymocyte development in mice lacking p56lck. , Published Online: 14 May 1992; | Doi:10.1038/357161a0 *357*, 161–164.

Mombaerts, P., Iacomini, J., Johnson, R.S., Herrup, K., Tonegawa, S., and Papaioannou, V.E. (1992). RAG-1-deficient mice have no mature B and T lymphocytes. Cell *68*, 869–877.

Montoya, M.C., Sancho, D., Vicente Manzanares, M., and Sánchez Madrid, F. (2002). Cell adhesion and polarity during immune interactions. Immunol. Rev. *186*, 68–82.

Mora, A.L., Chen, D., Boothby, M., and Rubin, D.H. (1999). Lineage-specific differences among CD8+ T cells in their dependence of NF-kappa B/Rel signaling. Eur. J. Immunol. *29*, 2968–2980.

Mora, A.L., Stanley, S., Armistead, W., Chan, A.C., and Boothby, M. (2001). Inefficient ZAP-70 phosphorylation and decreased thymic selection in vivo result from inhibition of NF-kappaB/Rel. The Journal of Immunology *167*, 5628–5635.

Mortazavi, A., Williams, B.A., McCue, K., Schaeffer, L., and Wold, B. (2008). Mapping and quantifying mammalian transcriptomes by RNA-Seq. Nat. Methods *5*, 621–628.

Murali-Krishna, K., Lau, L.L., Sambhara, S., Lemonnier, F., Altman, J., and Ahmed, R. (1999). Persistence of memory CD8 T cells in MHC class I-deficient mice. Science *286*, 1377–1381.

Murphy, K.M. (2011). Janeway's Immunobiology (Garland Science).

Naudé, P.J.W., Boer, den, J.A., Luiten, P.G.M., and Eisel, U.L.M. (2011). Tumor necrosis factor receptor cross-talk. FEBS Journal *278*, 888–898.

Nishikomori, R., Akutagawa, H., Maruyama, K., Nakata-Hizume, M., Ohmori, K., Mizuno, K., Yachie, A., Yasumi, T., Kusunoki, T., Heike, T., et al. (2004). X-linked ectodermal dysplasia and immunodeficiency caused by reversion

mosaicism of NEMO reveals a critical role for NEMO in human T-cell development and/or survival. Blood *103*, 4565–4572.

O'Donnell, M.A., Perez-Jimenez, E., Oberst, A., Ng, A., Massoumi, R., Xavier, R., Green, D.R., and Ting, A.T. (2011). Caspase 8 inhibits programmed necrosis by processing CYLD. Nat. Cell Biol. *13*, 1437–1442.

Oberst, A., Dillon, C.P., Weinlich, R., McCormick, L.L., Fitzgerald, P., Pop, C., Hakem, R., Salvesen, G.S., and Green, D.R. (2011). Catalytic activity of the caspase-8-FLIP(L) complex inhibits RIPK3-dependent necrosis. Nature *471*, 363–367.

Ochoa-Garay, J., Kaye, J., and Coligan, J.E. (1998). Nuclear factor kappaB is required for peptide antigen-induced differentiation of a CD4+CD8+ thymocyte line. The Journal of Immunology *160*, 3835–3843.

Oeckinghaus, A., Hayden, M.S., and Ghosh, S. (2011). Crosstalk in NF-κB signaling pathways. Nat Immunol *12*, 695–708.

Ofengeim, D., and Yuan, J. (2013). Regulation of RIP1 kinase signalling at the crossroads of inflammation and cell death. Nat. Rev. Mol. Cell Biol. *14*, 727–736.

Okada, T., Miller, M.J., Parker, I., Krummel, M.F., Neighbors, M., Hartley, S.B., O'Garra, A., Cahalan, M.D., and Cyster, J.G. (2005). Antigen-Engaged B Cells Undergo Chemotaxis toward the T Zone and Form Motile Conjugates with Helper T Cells. PLOS Biol *3*, e150.

Padovan, E., Casorati, G., and Dellabona, P. (1993). Expression of Two T Cell Receptor Chains: Dual Receptor T Cells. … -New York Then ….

Palmer, M.J., Mahajan, V.S., Trajman, L.C., Irvine, D.J., Lauffenburger, D.A., and Chen, J. (2008). Interleukin-7 receptor signaling network: an integrated systems perspective. Cell. Mol. Immunol. *5*, 79–89.

Palucka, K.A., Taquet, N., Sanchez-Chapuis, F., and Gluckman, J.C. (1998). Dendritic cells as the terminal stage of monocyte differentiation. The Journal of Immunology *160*, 4587–4595.

Park, J.-H., Yu, Q., Erman, B., Appelbaum, J.S., Montoya-Durango, D., Grimes, H.L., and Singer, A. (2004). Suppression of IL7Ralpha transcription by IL-7 and other prosurvival cytokines: a novel mechanism for maximizing IL-7-dependent T cell survival. Immunity *21*, 289–302.

Pasparakis, M., Luedde, T., and Schmidt-Supprian, M. (2006). Dissection of the NF-κB signalling cascade in transgenic and knockout mice. *13*, 861–872.

Pasparakis, M., and Vandenabeele, P. (2015). Necroptosis and its role in inflammation. Nature *517*, 311–320.

Paul, W.E., and Zhu, J. (2010). How are TH2-type immune responses initiated and amplified? Nat Rev Immunol *10*, 225–235.

Pauling, L., and Delbrück, M. (1940). The nature of the intermolecular forces operative in biological processes. Science *92*, 77–79.

Pawson, T., and Nash, P. (2003). Assembly of cell regulatory systems through protein interaction domains. Science *300*, 445–452.

Paxian, S., Merkle, H., Riemann, M., Wilda, M., Adler, G., Hameister, H., Liptay, S., Pfeffer, K., and Schmid, R.M. (2002). Abnormal organogenesis of Peyer's patches in mice deficient for NF-κB1, NF-κB2, and Bcl-3. Gastroenterology *122*, 1853–1868.

Pearse, G. (2006). Normal Structure, Function and Histology of the Thymus. Toxicol Pathol *34*, 504–514.

Peschon, J.J., Morrissey, P.J., Grabstein, K.H., Ramsdell, F.J., Maraskovsky, E., Gliniak, B.C., Park, L.S., Ziegler, S.F., Williams, D.E., Ware, C.B., et al. (1994). Early lymphocyte expansion is severely impaired in interleukin 7 receptor-deficient mice. Journal of Experimental Medicine *180*, 1955–1960.

Pobezinskaya, Y.L., Kim, Y.-S., Choksi, S., Morgan, M.J., Li, T., Liu, C., and Liu, Z. (2008). The function of TRADD in signaling through tumor necrosis factor receptor 1 and TRIF-dependent Toll-like receptors. Nat Immunol *9*, 1047–1054.

Polic, B., Kunkel, D., Scheffold, A., and Rajewsky, K. (2001). How alpha beta T cells deal with induced TCR alpha ablation. Pnas *98*, 8744–8749.

Pop, C., Oberst, A., Drag, M., Van Raam, B.J., Riedl, S.J., Green, D.R., and Salvesen, G.S. (2011). FLIP(L) induces caspase 8 activity in the absence of interdomain caspase 8 cleavage and alters substrate specificity. Biochem. J. *433*, 447–457.

Pozzesi, N., Fierabracci, A., Liberati, A.M., Martelli, M.P., Ayroldi, E., Riccardi, C., and Delfino, D.V. (2014). Role of caspase-8 in thymus function. *21*, 226–233.

Prasad, K.V., Ao, Z., Yoon, Y., Wu, M.X., Rizk, M., Jacquot, S., and Schlossman, S.F. (1997). CD27, a member of the tumor necrosis factor receptor family, induces apoptosis and binds to Siva, a proapoptotic protein. Pnas *94*, 6346–6351.

Prendes, M., Zheng, Y., and Beg, A.A. (2003). Regulation of developing B cell survival by RelA-containing NF-kappa B complexes. The Journal of Immunology *171*, 3963–3969.

Puri, K.D., Finger, E.B., Gaudernack, G., and Springer, T.A. (1995). Sialomucin CD34 is the major L-selectin ligand in human tonsil high endothelial venules. J Cell Biol *131*, 261–270.

Qian, Y., Commane, M., Ninomiya-Tsuji, J., Matsumoto, K., and Li, X. (2001). IRAK-mediated translocation of TRAF6 and TAB2 in the interleukin-1-induced activation of NFkappa B. Journal of Biological Chemistry *276*, 41661–41667.

Quah, B.J., and Parish, C.R. (2001). Innate Immune Mechanisms: Nonself Recognition (Chichester, UK: John Wiley & Sons, Ltd).

Radbruch, A., Muehlinghaus, G., Luger, E.O., Inamine, A., Smith, K.G.C., Dörner, T., and Hiepe, F. (2006). Competence and competition: the challenge of becoming a long-lived plasma cell. Nat Rev Immunol *6*, 741–750.

Ren, H., Schmalstieg, A., van Oers, N.S.C., and Gaynor, R.B. (2002). I-kappa B kinases alpha and beta have distinct roles in regulating murine T cell function. The Journal of Immunology *168*, 3721–3731.

Ritter, A.T., Angus, K.L., and Griffiths, G.M. (2013). The role of the cytoskeleton at the immunological synapse. Immunol. Rev. *256*, 107–117.

Roncarolo, M.G., Bacchetta, R., Bordignon, C., Narula, S., and Levings, M.K. (2001). Type 1 T regulatory cells. Immunol. Rev. *182*, 68–79.

Rosenfeld, M.E., Prichard, L., Shiojiri, N., and Fausto, N. (2000). Prevention of hepatic apoptosis and embryonic lethality in RelA/TNFR-1 double knockout mice. The American Journal of Pathology *156*, 997–1007.

Rossman, J.S., Stoicheva, N.G., Langel, F.D., Patterson, G.H., Lippincott-Schwartz, J., and Schaefer, B.C. (2006). POLKADOTS are foci of functional interactions in T-Cell receptor-mediated signaling to NF-kappaB. Mol. Biol. Cell *17*, 2166–2176.

Rothe, J., Lesslauer, W., Lötscher, H., Lang, Y., Koebel, P., Kontgen, F., Althage, A., Zinkernagel, R., Steinmetz, M., and Bluethmann, H. (1993). Mice lacking the tumour necrosis factor receptor 1 are resistant to TNF-mediated toxicity but highly susceptible to infection by Listeria monocytogenes. Nature *364*, 798–802.

Rudolph, D., Yeh, W.C., Wakeham, A., Rudolph, B., Nallainathan, D., Potter, J., Elia, A.J., and Mak, T.W. (2000). Severe liver degeneration and lack of NF-kappaB activation in NEMO/IKKgamma-deficient mice. Genes & Development *14*, 854–862.

Rueda, D., and Thome, M. (2005). Phosphorylation of CARMA1: the link(er) to NF-kappaB activation. Immunity *23*, 551–553.

Ruland, J., Duncan, G.S., Elia, A., del Barco Barrantes, I., Nguyen, L., Plyte, S., Millar, D.G., Bouchard, D., Wakeham, A., Ohashi, P.S., et al. (2001). Bcl10 is a positive regulator of antigen receptor-induced activation of NF-kappaB and neural tube closure. Cell *104*, 33–42.

Russell, J.H., and Ley, T.J. (2003). LYMPHOCYTE-MEDIATED CYTOTOXICITY. Http://Dx.Doi.org/10.1146/Annurev.Immunol.20.100201.131730 *20*, 323–370.

Rutemark, C., Bergman, A., Getahun, A., Hallgren, J., Henningsson, F., and Heyman, B. (2012). Complement Receptors 1 and 2 in Murine Antibody Responses to IgM-Complexed and Uncomplexed Sheep Erythrocytes. PLoS

ONE *7*, e41968.

Safa, A.R. (2013). Roles of c-FLIP in Apoptosis, Necroptosis, and Autophagy. J Carcinog Mutagen *Suppl 6*.

Saini, M., Sinclair, C., Marshall, D., Tolaini, M., Sakaguchi, S., and Seddon, B. (2010). Regulation of Zap70 Expression During Thymocyte Development Enables Temporal Separation of CD4 and CD8 Repertoire Selection at Different Signaling Thresholds. Science Signaling *3*, ra23–ra23.

Salmena, L., Lemmers, B., Hakem, A., Matysiak-Zablocki, E., Murakami, K., Au, P.Y.B., Berry, D.M., Tamblyn, L., Shehabeldin, A., Migon, E., et al. (2003). Essential role for caspase 8 in T-cell homeostasis and T-cell-mediated immunity. Genes & Development *17*, 883–895.

Samelson, L.E. (2003). SIGNAL TRANSDUCTION MEDIATED BY THE T CELL ANTIGEN RECEPTOR: The Role of Adapter Proteins*. Http://Dx.Doi.org/10.1146/Annurev.Immunol.20.092601.111357 *20*, 371–394.

Saraiva, M., and O'Garra, A. (2010). The regulation of IL-10 production by immune cells. Nat Rev Immunol *10*, 170–181.

Sato, S., Sanjo, H., Takeda, K., Ninomiya-Tsuji, J., Yamamoto, M., Kawai, T., Matsumoto, K., Takeuchi, O., and Akira, S. (2005). Essential function for the kinase TAK1 in innate and adaptive immune responses. Nat Immunol *6*, 1087–1095.

Sato, S., Sanjo, H., Tsujimura, T., Ninomiya-Tsuji, J., Yamamoto, M., Kawai, T., Takeuchi, O., and Akira, S. (2006). TAK1 is indispensable for development of T cells and prevention of colitis by the generation of regulatory T cells. International Immunology *18*, 1405–1411.

Sawicka, M., Stritesky, G.L., Reynolds, J., Abourashchi, N., Lythe, G., Molina-París, C., and Hogquist, K.A. (2014). From pre-DP, post-DP, SP4, and SP8 Thymocyte Cell Counts to a Dynamical Model of Cortical and Medullary Selection. Front Immunol *5*, 19.

Schatz, D.G., and Ji, Y. (2011). Recombination centres and the orchestration of V(D)J recombination. Nat Rev Immunol *11*, 251–263.

Schim van der Loeff, I., Hsu, L.-Y., Saini, M., Weiss, A., and Seddon, B. (2014). Zap70 Is Essential for Long-Term Survival of Naive CD8 T Cells. J. Immunol. 1400858.

Schluns, K.S., Kieper, W.C., Jameson, S.C., and Lefrançois, L. (2000). Interleukin-7 mediates the homeostasis of naïve and memory CD8 T cells in vivo. Nat Immunol *1*, 426–432.

Schmidt-Supprian, M., Bloch, W., Courtois, G., Addicks, K., Israel, A., Rajewsky, K., and Pasparakis, M. (2000). NEMO/IKK gamma-deficient mice model incontinentia pigmenti. Mol. Cell *5*, 981–992.

Schmidt-Supprian, M., Courtois, G., Tian, J., Coyle, A.J., Israel, A., Rajewsky, K., and Pasparakis, M. (2003). Mature T cells depend on signaling through the IKK complex. Immunity *19*, 377–389.

Schmidt-Supprian, M., Tian, J., Ji, H., Terhorst, C., Bhan, A.K., Grant, E.P., Pasparakis, M., Casola, S., Coyle, A.J., and Rajewsky, K. (2004). I kappa B kinase 2 deficiency in T cells leads to defects in priming, B cell help, germinal center reactions, and homeostatic expansion. The Journal of Immunology *173*, 1612–1619.

Schmitt, N., and Ueno, H. (2015). Regulation of human helper T cell subset differentiation by cytokines. Current Opinion in Immunology *34*, 130–136.

Schubert, L.A., Jeffery, E., Zhang, Y., Ramsdell, F., and Ziegler, S.F. (2001). Scurfin (FOXP3) acts as a repressor of transcription and regulates T cell activation. Journal of Biological Chemistry *276*, 37672–37679.

Schwartz, R.H. (2003). T cell anergy. Annu. Rev. Immunol. *21*, 305–334.

Seddon, B., Legname, G., Tomlinson, P., and Zamoyska, R. (2000). Long-term survival but impaired homeostatic proliferation of Naïve T cells in the absence of p56lck. Science *290*, 127–131.

Seddon, B., and Zamoyska, R. (2002). TCR and IL-7 receptor signals can operate independently or synergize to promote lymphopenia-induced expansion of naive T cells. The Journal of Immunology *169*, 3752–3759.

Seddon, B., Tomlinson, P., and Zamoyska, R. (2003). Interleukin 7 and T cell receptor signals regulate homeostasis of CD4 memory cells. Nat Immunol *4*, 680–686.

Sedger, L.M., and McDermott, M.F. (2014). TNF and TNF-receptors: From mediators of cell death and inflammation to therapeutic giants - past, present and future. Cytokine Growth Factor Rev. *25*, 453–472.

Sen, R., and Baltimore, D. (1986). Inducibility of kappa immunoglobulin enhancer-binding protein Nf-kappa B by a posttranslational mechanism. Cell *47*, 921–928.

Senftleben, U., Cao, Y., Xiao, G., Greten, F.R., Krähn, G., Bonizzi, G., Chen, Y., Hu, Y., Fong, A., Sun, S.C., et al. (2001a). Activation by IKKalpha of a second, evolutionary conserved, NF-kappa B signaling pathway. Science *293*, 1495–1499.

Senftleben, U., Li, Z.-W., Baud, V., and Karin, M. (2001b). IKKβ Is Essential for Protecting T Cells from TNFα-Induced Apoptosis. Immunity *14*, 217–230.

Serwold, T., Hochedlinger, K., Inlay, M.A., Jaenisch, R., and Weissman, I.L. (2007). Early TCR expression and aberrant T cell development in mice with endogenous prerearranged T cell receptor genes. The Journal of Immunology *179*, 928–938.

Sha, W.C., Liou, H.C., Tuomanen, E.I., and Baltimore, D. (1995). Targeted disruption of the p50 subunit of NF-kappa B leads to multifocal defects in immune responses. Cell *80*, 321–330.

Shakhov, A.N., Collart, M.A., Vassalli, P., Nedospasov, S.A., and Jongeneel, C.V. (1990). Kappa B-type enhancers are involved in lipopolysaccharide-mediated transcriptional activation of the tumor necrosis factor alpha gene in primary macrophages. Journal of Experimental Medicine *171*, 35–47.

Shim, J.-H., Xiao, C., Paschal, A.E., Bailey, S.T., Rao, P., Hayden, M.S., Lee, K.-Y., Bussey, C., Steckel, M., Tanaka, N., et al. (2005). TAK1, but not TAB1 or TAB2, plays an essential role in multiple signaling pathways in vivo. Genes & Development *19*, 2668–2681.

Shimshek, D.R., Kim, J., Hübner, M.R., Spergel, D.J., Buchholz, F., Casanova, E., Stewart, A.F., Seeburg, P.H., and Sprengel, R. (2002). Codon-improved Cre recombinase (iCre) expression in the mouse. Genesis *32*, 19–26.

Shortman, K., and Liu, Y.-J. (2002). Mouse and human dendritic cell subtypes. Nat Rev Immunol *2*, 151–161.

Siegel, R.M. (2006). Caspases at the crossroads of immune-cell life and death. Nat Rev Immunol *6*, 308–317.

Silke, J. (2011). The regulation of TNF signalling: what a tangled web we weave. Current Opinion in Immunology *23*, 620–626.

Silva, A., Cornish, G., Ley, S.C., and Seddon, B. (2014). NF-κB signaling mediates homeostatic maturation of new T cells. Proc. Natl. Acad. Sci. U.S.a. *111*, E846–E855.

Silverstein, A.M. (2012). A History of Immunology (Elsevier).

Sinclair, C., Saini, M., Schim van der Loeff, I., Sakaguchi, S., and Seddon, B. (2011). The Long-Term Survival Potential of Mature T Lymphocytes Is Programmed During Development in the Thymus. Science Signaling *4*, ra77–ra77.

Sinclair, C., and Seddon, B. (2014). Overlapping and asymmetric functions of TCR signaling during thymic selection of CD4 and CD8 lineages. J. Immunol. *192*, 5151–5159.

Sinclair, C., Bains, I., Yates, A.J., and Seddon, B. (2013). Asymmetric thymocyte death underlies the CD4:CD8 T-cell ratio in the adaptive immune system. Proc. Natl. Acad. Sci. U.S.a. *110*, E2905–E2914.

Sinclair, C., Ono, M., and Seddon, B. (2015). A Zap70-dependent feedback circuit is essential for efficient selection of CD4 lineage thymocytes. Immunol Cell Biol *93*, 406–416.

Singer, A., Adoro, S., and Park, J.-H. (2008). Lineage fate and intense debate: myths, models and mechanisms of CD4- versus CD8-lineage choice. Nat Rev

Immunol *8*, 788–801.

Slauch, J.M. (2011). How does the oxidative burst of macrophages kill bacteria? Still an open question. Molecular Microbiology *80*, 580–583.

Smith-Garvin, J.E., Koretzky, G.A., and Jordan, M.S. (2009). T cell activation. Annu. Rev. Immunol. *27*, 591–619.

Snapper, C.M., Rosas, F.R., Zelazowski, P., Moorman, M.A., Kehry, M.R., Bravo, R., and Weih, F. (1996). B cells lacking RelB are defective in proliferative responses, but undergo normal B cell maturation to Ig secretion and Ig class switching. Journal of Experimental Medicine *184*, 1537–1541.

Sohn, S.J., Forbush, K.A., Nguyen, N., Witthuhn, B., Nosaka, T., Ihle, J.N., and Perlmutter, R.M. (1998). Requirement for Jak3 in mature T cells: its role in regulation of T cell homeostasis. The Journal of Immunology *160*, 2130–2138.

Spiegel, S., and Milstien, S. (2011). The outs and the ins of sphingosine-1-phosphate in immunity. Nat Rev Immunol *11*, 403–415.

Srinivas, S., Watanabe, T., Lin, C.S., William, C.M., Tanabe, Y., Jessell, T.M., and Costantini, F. (2001). Cre reporter strains produced by targeted insertion of EYFP and ECFP into the ROSA26 locus. BMC Dev. Biol. *1*, 4.

Sriskantharajah, S., Belich, M.P., Papoutsopoulou, S., Janzen, J., Tybulewicz, V., Seddon, B., and Ley, S.C. (2008). Proteolysis of NF-κB1 p105 is essential for T cell antigen receptor–induced proliferation. Nat Immunol *10*, 38–47.

Stehlik, C., de Martin, R., Kumabashiri, I., Schmid, J.A., Binder, B.R., and Lipp, J. (1998). Nuclear factor (NF)-kappaB-regulated X-chromosome-linked iap gene expression protects endothelial cells from tumor necrosis factor alpha-induced apoptosis. Journal of Experimental Medicine *188*, 211–216.

Stoven, S., Silverman, N., Junell, A., Hedengren-Olcott, M., Erturk, D., Engstrom, Y., Maniatis, T., and Hultmark, D. (2003). Caspase-mediated processing of the Drosophila NF-kappaB factor Relish. Pnas *100*, 5991–5996.

Strasser, A. (2005). The role of BH3-only proteins in the immune system. Nat Rev Immunol *5*, 189–200.

Su, H., Bidère, N., Zheng, L., Cubre, A., Sakai, K., Dale, J., Salmena, L., Hakem, R., Straus, S., and Lenardo, M. (2005). Requirement for caspase-8 in NF-kappaB activation by antigen receptor. Science *307*, 1465–1468.

Sudo, T., Nishikawa, S., Ohno, N., Akiyama, N., Tamakoshi, M., and Yoshida, H. (1993). Expression and function of the interleukin 7 receptor in murine lymphocytes. Proc. Natl. Acad. Sci. U.S.a. *90*, 9125–9129.

Sumaria, N., Roediger, B., Ng, L.G., Qin, J., Pinto, R., Cavanagh, L.L., Shklovskaya, E., Fazekas de St Groth, B., Triccas, J.A., and Weninger, W. (2011). Cutaneous immunosurveillance by self-renewing dermal T cells. Journal of Experimental Medicine *208*, 505–518.

Sun, G., Liu, X., Mercado, P., Jenkinson, S.R., Kypriotou, M., Feigenbaum, L., Galéra, P., and Bosselut, R. (2005). The zinc finger protein cKrox directs CD4 lineage differentiation during intrathymic T cell positive selection. Nat Immunol *6*, 373–381.

Sun, L., Deng, L., Ea, C.-K., Xia, Z.-P., and Chen, Z.J. (2004). The TRAF6 ubiquitin ligase and TAK1 kinase mediate IKK activation by BCL10 and MALT1 in T lymphocytes. Mol. Cell *14*, 289–301.

Sun, S.C. (2010). CYLD: a tumor suppressor deubiquitinase regulating NF-kappaB activation and diverse biological processes. *17*, 25–34.

Swain, S.L. (2000). CD4 T–cell memory can persist in the absence of class II. Philosophical Transactions of the Royal Society of London B: Biological Sciences *355*, 407–411.

Szabo, S.J., Sullivan, B.M., Peng, S.L., and Glimcher, L.H. (2003). Molecular mechanisms regulating Th1 immune responses. Annu. Rev. Immunol. *21*, 713–758.

Tada, K., Okazaki, T., Sakon, S., Kobarai, T., Kurosawa, K., Yamaoka, S., Hashimoto, H., Mak, T.W., Yagita, H., Okumura, K., et al. (2001). Critical roles of TRAF2 and TRAF5 in tumor necrosis factor-induced NF-kappa B activation and protection from cell death. Journal of Biological Chemistry *276*, 36530–36534.

Takada, K., and Jameson, S.C. (2009). Naive T cell homeostasis: from awareness of space to a sense of place. Nat Rev Immunol *9*, 823–832.

Takahama, Y. (2006). Journey through the thymus: stromal guides for T-cell development and selection. Nat Rev Immunol *6*, 127–135.

Takeda, K., Takeuchi, O., Tsujimura, T., Itami, S., Adachi, O., Kawai, T., Sanjo, H., Yoshikawa, K., Terada, N., and Akira, S. (1999). Limb and skin abnormalities in mice lacking IKKalpha. Science *284*, 313–316.

Takeda, K., and Akira, S. (2015). Toll-like receptors. Curr Protoc Immunol *109*, 14.12.1–14.12.10.

Tanaka, M., Fuentes, M.E., Yamaguchi, K., Durnin, M.H., Dalrymple, S.A., Hardy, K.L., and Goeddel, D.V. (1999). Embryonic lethality, liver degeneration, and impaired NF-kappa B activation in IKK-beta-deficient mice. Immunity *10*, 421–429.

Tato, C.M., Villarino, A., Caamaño, J.H., Boothby, M., and Hunter, C.A. (2003). Inhibition of NF-kappa B activity in T and NK cells results in defective effector cell expansion and production of IFN-gamma required for resistance to Toxoplasma gondii. The Journal of Immunology *170*, 3139–3146.

Thome, M. (2004). CARMA1, BCL-10 and MALT1 in lymphocyte development and activation. Nat Rev Immunol *4*, 348–359.

Thompson, M.R., Kaminski, J.J., Kurt-Jones, E.A., and Fitzgerald, K.A. (2011). Pattern recognition receptors and the innate immune response to viral infection. Viruses *3*, 920–940.

Tian, T., Zhang, J., Gao, L., Qian, X.P., and Chen, W.F. (2001). Heterogeneity within medullary-type TCRalphabeta(+)CD3(+)CD4(-)CD8(+) thymocytes in normal mouse thymus. International Immunology *13*, 313–320.

Uematsu, Y., Ryser, S., Dembić, Z., Borgulya, P., Krimpenfort, P., Berns, A., Boehmer, von, H., and Steinmetz, M. (1988). In transgenic mice the introduced functional T cell receptor beta gene prevents expression of endogenous beta genes. Cell *52*, 831–841.

Valitutti, S., Dessing, M., Aktories, K., Gallati, H., and Lanzavecchia, A. (1995). Sustained signaling leading to T cell activation results from prolonged T cell receptor occupancy. Role of T cell actin cytoskeleton. Journal of Experimental Medicine *181*, 577–584.

Van Antwerp, D.J., Martin, S.J., Kafri, T., Green, D.R., and Verma, I.M. (1996). Suppression of TNF-alpha-induced apoptosis by NF-kappaB. Science *274*, 787–789.

Vanden Berghe, T., Linkermann, A., Jouan-Lanhouet, S., Walczak, H., and Vandenabeele, P. (2014). Regulated necrosis: the expanding network of non-apoptotic cell death pathways. Nat. Rev. Mol. Cell Biol. *15*, 135–147.

Vandenabeele, P., Declercq, W., Beyaert, R., and Fiers, W. (1995). Two tumour necrosis factor receptors: structure and function. Trends Cell Biol. *5*, 392–399.

Varfolomeev, E.E., and Ashkenazi, A. (2004). Tumor necrosis factor: an apoptosis JuNKie? Cell *116*, 491–497.

Varfolomeev, E., Goncharov, T., Fedorova, A.V., Dynek, J.N., Zobel, K., Deshayes, K., Fairbrother, W.J., and Vucic, D. (2008). c-IAP1 and c-IAP2 are critical mediators of tumor necrosis factor alpha (TNFalpha)-induced NF-kappaB activation. Journal of Biological Chemistry *283*, 24295–24299.

Vella, A.T., Dow, S., Potter, T.A., Kappler, J., and Marrack, P. (1998). Cytokine-induced survival of activated T cells in vitro and in vivo. Pnas *95*, 3810–3815.

Voll, R.E., Jimi, E., Phillips, R.J., Barber, D.F., Rincon, M., Hayday, A.C., Flavell, R.A., and Ghosh, S. (2000). NF-kappa B activation by the pre-T cell receptor serves as a selective survival signal in T lymphocyte development. Immunity *13*, 677–689.

Wan, Y.Y., Chi, H., Xie, M., Schneider, M.D., and Flavell, R.A. (2006). The kinase TAK1 integrates antigen and cytokine receptor signaling for T cell development, survival and function. Nat Immunol *7*, 851–858.

Wang, C.Y., Mayo, M.W., and Baldwin, A.S. (1996). TNF- and cancer therapy-induced apoptosis: potentiation by inhibition of NF-kappaB. Science *274*, 784–787.

Wang, C.Y., Mayo, M.W., Korneluk, R.G., Goeddel, D.V., and Baldwin, A.S. (1998). NF-kappaB antiapoptosis: induction of TRAF1 and TRAF2 and c-IAP1 and c-IAP2 to suppress caspase-8 activation. Science *281*, 1680–1683.

Wang, C., Deng, L., Hong, M., Akkaraju, G.R., Inoue, J., and Chen, Z.J. (2001). TAK1 is a ubiquitin-dependent kinase of MKK and IKK. Nature *412*, 346–351.

Wang, D., You, Y., Case, S.M., McAllister-Lucas, L.M., Wang, L., DiStefano, P.S., Nuñez, G., Bertin, J., and Lin, X. (2002). A requirement for CARMA1 in TCR-induced NF-kappa B activation. Nat Immunol *3*, 830–835.

Wang, L., Du, F., and Wang, X. (2008). TNF-alpha induces two distinct caspase-8 activation pathways. Cell *133*, 693–703.

Watts, T.H. (2004). TNF/TNFR Family Members in Costimulation of T Cell Responses. Http://Dx.Doi.org/10.1146/Annurev.Immunol.23.021704.115839 *23*, 23–68.

Weinreich, M.A., Jameson, S.C., and Hogquist, K.A. (2011). Postselection thymocyte maturation and emigration are independent of IL-7 and ERK5. J. Immunol. *186*, 1343–1347.

Weninger, W., Manjunath, N., and Andrian, Von, U.H. (2002). Migration and differentiation of CD8+ T cells. Immunol. Rev. *186*, 221–233.

Wilson, A., MacDonald, H.R., and Radtke, F. (2001). Notch 1-deficient common lymphoid precursors adopt a B cell fate in the thymus. Journal of Experimental Medicine *194*, 1003–1012.

Wong, W.W.-L., Gentle, I.E., Nachbur, U., Anderton, H., Vaux, D.L., and Silke, J. (2010). RIPK1 is not essential for TNFR1-induced activation of NF-kappaB. *17*, 482–487.

Wu, H., Tschopp, J., and Lin, S.-C. (2007). Smac Mimetics and TNFα: A Dangerous Liaison? Cell *131*, 655–658.

Wu, L., and Liu, Y.-J. (2007). Development of dendritic-cell lineages. Immunity *26*, 741–750.

Wu, M., Lee, H., Bellas, R.E., Schauer, S.L., Arsura, M., Katz, D., FitzGerald, M.J., Rothstein, T.L., Sherr, D.H., and Sonenshein, G.E. (1996). Inhibition of NF-kappaB/Rel induces apoptosis of murine B cells. Embo J. *15*, 4682–4690.

Wu, Y., Borde, M., Heissmeyer, V., Feuerer, M., Lapan, A.D., Stroud, J.C., Bates, D.L., Guo, L., Han, A., Ziegler, S.F., et al. (2006). FOXP3 Controls Regulatory T Cell Function through Cooperation with NFAT. Cell *126*, 375–387.

Wucherpfennig, K.W., Gagnon, E., Call, M.J., Huseby, E.S., and Call, M.E. (2010). Structural Biology of the T-cell Receptor: Insights into Receptor Assembly, Ligand Recognition, and Initiation of Signaling. Cold Spring Harbor Perspectives in Biology *2*, a005140–a005140.

Xiao, G., Harhaj, E.W., and Sun, S.C. (2001). NF-kappaB-inducing kinase regulates the processing of NF-kappaB2 p100. Mol. Cell *7*, 401–409.

Xiong, J., Armato, M.A., and Yankee, T.M. (2011). Immature single-positive CD8+ thymocytes represent the transition from Notch-dependent to Notch-independent T-cell development. International Immunology *23*, 55–64.

Yamamoto, Y., Yin, M.J., and Gaynor, R.B. (2000). IkappaB kinase alpha (IKKalpha) regulation of IKKbeta kinase activity. Mol. Cell. Biol. *20*, 3655–3666.

Yan, J., Xiang, J., Lin, Y., Ma, J., Zhang, J., Zhang, H., Sun, J., Danial, N.N., Liu, J., and Lin, A. (2013). Inactivation of BAD by IKK inhibits TNFα-induced apoptosis independently of NF-κB activation. Cell *152*, 304–315.

Yasukawa, H., Sasaki, A., and Yoshimura, A. (2000). Negative regulation of cytokine signaling pathways. Annu. Rev. Immunol. *18*, 143–164.

Yeh, W.C., Shahinian, A., Speiser, D., Kraunus, J., Billia, F., Wakeham, A., la Pompa, de, J.L., Ferrick, D., Hum, B., Iscove, N., et al. (1997). Early lethality, functional NF-kappaB activation, and increased sensitivity to TNF-induced cell death in TRAF2-deficient mice. Immunity *7*, 715–725.

Yilmaz, Z.B., Weih, D.S., Sivakumar, V., and Weih, F. (2003). RelB is required for Peyer's patch development: differential regulation of p52–RelB by lymphotoxin and TNF. Embo J. *22*, 121–130.

Yin, Q., Lamothe, B., Darnay, B.G., and Wu, H. (2009). Structural basis for the lack of E2 interaction in the RING domain of TRAF2. Biochemistry *48*, 10558–10567.

Zhang, N., and He, Y.-W. (2005). An essential role for c-FLIP in the efficient development of mature T lymphocytes. Journal of Experimental Medicine *202*, 395–404.

Zhang, S.Q., Kovalenko, A., Cantarella, G., and Wallach, D. (2000). Recruitment of the IKK signalosome to the p55 TNF receptor: RIP and A20 bind to NEMO (IKKgamma) upon receptor stimulation. Immunity *12*, 301–311.

Zhang, Y., Rosenberg, S., Wang, H., Imtiyaz, H.Z., Hou, Y.-J., and Zhang, J. (2005). Conditional Fas-associated death domain protein (FADD): GFP knockout mice reveal FADD is dispensable in thymic development but essential in peripheral T cell homeostasis. The Journal of Immunology *175*, 3033–3044.

Zheng, Y., Vig, M., Lyons, J., Van Parijs, L., and Beg, A.A. (2003). Combined Deficiency of p50 and cRel in CD4+ T Cells Reveals an Essential Requirement for Nuclear Factor B in Regulating Mature T Cell Survival and In Vivo Function. Journal of Experimental Medicine *197*, 861–874.

Zhumabekov, T., Corbella, P., Tolaini, M., and Kioussis, D. (1995). Improved version of a human CD2 minigene based vector for T cell-specific expression in transgenic mice. J. Immunol. Methods *185*, 133–140.

Zola, H., Swart, B., Nicholson, I., Aasted, B., Bensussan, A., Boumsell, L., Buckley, C., Clark, G., Drbal, K., Engel, P., et al. (2005). CD molecules 2005: human cell differentiation molecules. Blood *106*, 3123–3126.